LAB. EXPTS. IN BIOLOGICAL CHEMISTRY

SUMNER

Laboratory Experiments In Biological Chemistry

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Second Edition, Revised and Enlarged

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PREFACE TO SECOND EDITION

In preparing a new edition of this laboratory manual we have continued the theme of the first edition — to provide fundamental training in laboratory biochemistry to students in any field of study. This edition differs from the earlier one principally in two respects. Recent improvements in apparatus and techniques have been included and new experiments have been added which reflect recent advancements in biochemistry. To make room for new experiments it has been necessary to eliminate some experiments given in the first edition. Practically all of the quantitative procedures involving colorimetric measurements have been written for the use of a photoelectric colorimeter. Since various types of photoelectric colorimeters are in use in different laboratories it may be necessary for the instructor to provide supplementary instructions applicable to the instrument available.

We wish to express our gratitude to our colleagues for various comments and criticisms. In particular we wish to express our appreciation to Dr. Walter L. Nelson for his worthwhile suggestions.

James B. Sumner.
G. Fred Somers.

CONTENTS

Laboratory Experiments In Biological Chemistry

GENERAL INSTRUCTIONS

Desk Reagents

The upper shelf includes: acetic acid, Barföd's reagent, Benedict's qualitative sugar reagent, biuret reagent, Lugol's solution, and dinitrosalicylic acid.

The lower shelf includes: concentrated hydrochloric acid (36% w/w), concentrated nitric acid (70%), 10 per cent sulfuric acid, solid ammonium sulfate, solid sodium chloride, 2 *N* ammonium hydroxide, 70 per cent sodium hydroxide.

Each bottle and glass stopper is numbered for each individual place. Do not let the bottles stray to other desks. Do not mix the stoppers. You are supposed to regard these reagents as a part of your desk equipment and to keep the bottles clean and in order.

General Qualitative Reagents

The reagents for qualitative work are in the cabinet or on the nearby shelf. They are all numbered and the number of each is given in the alphabetical list.

General Quantitative Reagents

Reagents for quantitative work are all on the shelf for quantitative reagents.

Notebooks

The student is expected to buy himself a blank book in which to record the results of the laboratory experiments, conclusions, calculations, drawings of apparatus, chemical reactions involved, etc. The book must not be loose-leaf. It is highly important that the results should be written in the book as they are obtained and not scribbled on scraps of paper with the intention of entering them later. The notebook should be ready for inspection at any time. Credit will be deducted if it is not written up. Quantitative experiments should be calculated as soon as finished.

Safety First

1. Do not work with ether, petroleum ether, benzene, alcohol, or other inflammable liquids in the vicinity of a flame.

2. Do not pipette strong acids, or alkalis, or poisonous compounds such as cyanide, or phenylhydrazine. When it is necessary to measure out strong acids or bases a graduated cylinder should be used. Cyanide is to be drawn from a burette. Phenylhydrazine is delivered from a pipette arranged so that it can be filled by immersing it in the reagent.

Do not suck pipettes placed in general laboratory reagents as it is not hygienic. These pipettes are meant to be filled by immersing them. After filling, they are stoppered by the finger, lifted out, and allowed to deliver.

3. Mix strong, strongly reactive, and unfamiliar reagents cautiously.

4. When boiling liquids in test tubes, shake the tube constantly (to prevent superheating) and keep it inclined away from your face and the faces of other persons.

5. Conduct all operations involving the production of fumes or bad odors under the hood. The mucic acid test, micro-Kjeldahl, and total sulfur determination should be done in the hood. Don't boil strong acids at your desks.

6. Always rinse the hands in water after pouring concentrated alkalies or acids from bottles.

7. Do not use, or leave about the laboratory, cracked, or jaggedly broken, glassware. Throw it into the slop jar at once. There is more to lose through getting badly cut than there is to gain by saving such glassware, which is always a menace.

The Toxicity of Certain Gases. It is not usually emphasized in chemical courses that certain gases such as hydrogen sulfide and nitric dioxide are extremely poisonous. The vapors of benzene, toluene, acetone, chloroform, mercury, and many other substances, are also poisonous. Symptoms may not become evident for several months.

Laboratory Rules

1. Do not throw solids (fat, matches, filter paper, etc.) into the sinks or troughs, or the sinks will become stopped up.

2. When pouring strong acids into the troughs or sinks, let the water run to wash the acid away.

3. Do not take reagents for general use to your desks. Others will be unable to find them.

4. Replace each reagent where it belongs and do not mix the stoppers.

5. Do not move pinchcocks of alcohol or distilled water so that the liquids are left dripping.

6. Try not to spill chemicals on the desks, floors, balances, colorimeters, microscopes, etc., and if you accidentally do so, mop the spilled material up at once.

7. Do not put pipettes into bottles containing quantitative reagents. Pour approximately the amount you need into a separate, clean, *dry* container and pipette from this. Discard the remainder.

Washing Apparatus

Soap, or one of the newer synthetic detergents, is the best of all cleansing agents when applied with hot water and plenty of rubbing with a test tube brush, sponge, or rag. Always wash a piece of apparatus first with soap before treating it with cleaning solution (chromic acid in sulfuric acid). This will save both time and cleaning solution. The apparatus used for qualitative work does not require cleaning with chromic acid, but should, nevertheless, be cleaned well with soap. However, students seldom use enough soap. A glance through the laboratory usually shows that most of the test tubes and apparatus in use are greasy and generally dirty. When the instructor wishes to repeat a test that some student has failed to obtain, he is likely to be handed one of these dirty test tubes.

After washing with soap, rinse well with tap water. The tap water should be drained off as thoroughly as possible before applying cleaning solution, as water dilutes cleaning solution and spoils it.

Cleaning solution should be used only for cleaning glassware to be used in quantitative work.

Preparation of Cleaning Solution: Danger

To about 85 g. of sodium dichromate add 100 cc. of water and boil until dissolved. Pour into a liter flask and cool rapidly by shaking under a stream of cold tap water. When the material is well cooled, place in the hood, and with great care add, slowly and with occasional rotation, 500 ml. of commerical sulfuric acid. Do not try to remove the hot solution until it has cooled. Let it stay in the hood for nearly an hour.

Another way of preparing cleaning solution is by adding finely ground sodium dichromate to concentrated sulfuric acid. Add only a small amount at a time and stir well after each addition.

Do Not Spill Cleaning Solution

It is extremely corrosive and destroys the floor, the soles of the shoes, the soapstone table tops, clothes, and many other materials. If cleaning solution is accidentally spilled, wash it off at once. If necessary, the spot can be washed with sodium carbonate solution. Do not suck cleaning solution up into pipettes. This is too dangerous. Clean your pipettes by immersing in cleaning solution contained in a long cylinder.

Do Not Place Cleaning Solution in Flasks or Pipettes that Contain Alcohol

Traces of alcohol immediately reduce the chromic acid in the cleaning solution to the green chromic sulfate. This ruins the cleaning solution. It has lost its oxidizing power. To convince yourself of this, add 2 ml. of cleaning solution to a few drops of alcohol contained in a test tube. Mix and observe the change in color.

Percentage Concentration of Solutions

The concentration, or quantitative value, or a solution, is usually expressed as so many "per cent" (%), which simply means "per hundred." The expression usually means parts of dissolved substance, usually expressed as grams (g.) per 100 ml. of solution. To prepare a 5 per cent solution of sodium chloride in water, one dissolves 5 g. of sodium chloride in a little water and then adds enough more water to make the total volume 100 ml. Then the solution is mixed thoroughly.

Sometimes the concentration of a solution is expressed "per mille" (‰), which simply means "per thousand," or per liter, since one liter contains 1000 ml.* To change grams per liter into per cent one divides by 10. Thus, 10 grams per liter (10 g./l.) is 1 per cent. One gamma (γ) is the same as 0.001 mg.

It is convenient to remember that grams per liter is the same thing as milligrams per ml. Thus, 10 grams per liter means 10 mg. per ml.

The concentration of solutions of alcohol is customarily expressed, not as grams of alcohol per 100 ml. of solution, but as volumes: *i.e.*, cubic centimeters. Thus, 60 per cent alcohol means that 100 ml. of the solution contains 60 ml. of pure alcohol.

Warning Concerning Use of Volatile Solvents

Several volatile solvents are used during this course. It is absolutely essential to understand the dangers that are connected with their use. These dangers are principally that of *Fire* and that of *Poisoning by inhalation. Do not spill volatile solvents around carelessly.* If solvent is spilled, wipe it up immediately. *The danger of fire cannot be overemphasized.* In this course all extractions and precipitations with volatile solvents are to be performed *in the hood with fan running, and no flames lighted.* When evaporating small amounts of volatile

* *Note:* The true liter (l.) is the volume occupied by 1 kilogram (Kg.) of pure water at 4.0° C. The correct designation for one-thousandth of this amount is a milliliter (ml.). Although the term cubic centimeter (cc.) has become habitual the term milliliter is to be preferred.

solvents, use the hot plate in the hood with the fan running. Do not allow material to boil over.

Most of the volatile solvents *cause poisoning by inhalation.* Acute poisoning can be caused by inhalation of moderately large amounts, but the greatest danger in the laboratory is inhalation of small amounts over a long period of time. Serious damage to liver and kidneys, for example, can result by such inhalation of dioxane and glycol ethers. Benzene and toluene may cause serious reduction in white cells of the blood. Chloroform, aside from its own poisonous action, may contain small amounts of carbonyl chloride (phosgene, the war gas). Carbon tetrachloride is poisonous itself, and may decompose to phosgene on hot surfaces. Carbon disulfide is particularly inflammable and poisonous.

We do not think there is great danger of acute poisoning by inhalation in this course, but unsuspected chronic poisoning may easily result by careless use of the solvents.

Preliminary Training in Analytical Chemistry

Before undertaking the quantitative experiments the student should acquaint himself with the fundamental operations involved in this sort of work. The student must be familiar with such procedures as the following:

1. The use and care of the analytical balance.
2. The use of pipettes.
3. The calibration of pipettes and burettes.
4. Titration.
5. The theory and use of indicators.
6. The preparation of solutions of different normalities.
7. Stoichiometrical calculations.
8. The significance of the decimal place in expressing results.
9. The calculation of the percentage error.
10. The method of making up to a known volume, filtering from a precipitate and taking an aliquot of the filtrate.
11. The dilution of 95 per cent alcohol to other percentages.
12. Discrimination in the use of dry flasks for some operations, and of wet flasks for others.

The student must not use his thumb on the pipette; must not attempt to measure 1 ml. of a quantitative solution in a wet, dirty 100 ml. graduate, or any other kind of graduated cylinder; must not

blow out the kind of pipette that should be allowed to drain; must not stick wet pipettes into quantitative solutions; must not use filter paper several sizes larger or smaller than the filtration funnel; must not mix up the stoppers of the bottles containing the quantitative reagents; must read burettes beyond the first decimal place; must use the analytical balance with care; must not pick up accurate weights with his fingers; must not spill chemicals on the weights, or on the pans of the balance, etc.

The Calculation of Results. The student is expected to calculate his analytical results by the use of logic, starting from normality values for the solutions used, from colorimetric readings, or from atomic weights. Nothing is so undesirable as the use of formulas for figuring out results, at least where elementary students are concerned.

All calculations should be made immediately after the analytical data are obtained and should be written in full in the notebook in such a manner that the instructor can see at a glance to what the figures refer.

Some Hints on the Technique of Volumetric Analysis

How can one dilute a 10 per cent solution to a 5 per cent solution?

Method A. With a clean, *dry* (see note below) 50 ml. pipette place 50 ml. of the 10 per cent solution in a clean (but not necessarily dry) 100 ml. volumetric flask. Now add water exactly to the 100 ml. graduation, stopper with a clean cork, and mix thoroughly.

Method B. Pipette 50 ml. of the 10 per cent solution into a clean and *dry* Erlenmeyer flask. Now add with a second pipette (the first pipette can be used if first well washed with water) exactly 50 ml. of water and mix well.

Note: After cleaning the pipette it can be dried by rinsing with alcohol and removing the alcohol by means of the compressed air, or by sucking air through the pipette. Another procedure is to wipe the outside of the wet pipette with a piece of *clean* cheesecloth and to rinse the inside twice with the solution which is to be pipetted. The rinsings are, of course, discarded. This last method is wasteful.

The Ostwald-Folin Pipette. This pipette, designed by Wilhelm Ostwald, was improved and made practical by Folin. It is especially adapted for the measurements of 1, 2, or 3 ml. portions of liquid where considerable accuracy is required. Measurement of 1 ml. of a solution by the Ostwald-Folin pipette can be made nearly 10 times more ac-

curately than by the ordinary 1 cc. transfer pipette. The student must always employ the Ostwald-Folin pipette (for 1, 2, or 3 ml.) when it is necessary to be as accurate as possible.

In filling, the liquid is sucked slowly up until it has passed above the graduation mark. Always use the index finger instead of the thumb to cap the end of the pipette. Now allow the liquid to drain until the bottom of the meniscus rests on the graduation mark. Avoid parallax in doing this. Now carefully wipe off the liquid that is suspended from the lower tip of the pipette, either by touching the tip to the side of a beaker, or test tube, or else by using a clean piece of cotton. Now the pipette is allowed to drain into the receptacle which is to receive the liquid. Ostwald pipettes are usually calibrated to be blown out and if this is the case let the pipette drain, holding the lower tip against the glass of the receiver, for 10 seconds after the liquid has ceased to run out, then blow once and drag the lower tip of the pipette against the receiver and remove the pipette. Pipettes that are calibrated to be blown out usually have two rings of ground glass near the top of the stem, or a ground glass band at the end of the stem.

Pipettes that are not calibrated to be blown out should be allowed to drain, holding the lower tip against the glass receiver. When the last of the liquid has drained out, rub the tip once against the glass and withdraw the pipette.

In some cases it is not possible to decide by inspection whether a pipette has been calibrated to blow out or to drain. In order to be sure one must calibrate the pipette. It is sometimes necessary to calibrate pipettes in order to make certain that they are as accurate as the manufacturer claims. Accordingly, the student is asked to calibrate his two 1 ml. Ostwald-Folin pipettes, using the procedure described below.

Calibration of Pipettes. From a flask of distilled water which is at nearly room temperature (measure the temperature!) fill the pipette and allow to drain (as has been described in the preceding article) into an accurately weighed small Erlenmeyer flask, or weighed bottle. The pipette must be absolutely clean, though not necessarily dry. Now weigh the container again as accurately as possible. The difference in weight is the apparent weight of the water delivered. To obtain the true weight, it would be necessary to make corrections for the buoyant effect of the air on the water, on the glass container, and on the weights. However, it is not necessary to do this if one uses the table given below,

which tells the apparent weight of 1 milliliter of water at different temperatures centigrade. Immediately determine the temperature of the water that you employed to calibrate your pipette. Now, by referring to the table, find the apparent weight of 1 milliliter of water at this temperature. Compare with this figure the weight of water delivered by your pipette. How great is the difference? As an example, let us suppose that a 1 ml. pipette delivers water at 20° C. that has an apparent weight of 0.9963 g. Since 1 ml. of water at 20° C. has an apparent weight of 0.9973 g. the error in this instance is 0.001, or one-tenth of one per cent, or one part in a thousand. This would be sufficiently accurate.

Apparent Weight of 1 ml. of Water at Different Temperatures Centrigrade

Temp. of Water Centigrade	Apparent wt. of 1 ml. of Water (Using Brass Weights)
15° C.	0.9980 g.
16° "	0.9979 "
17° "	0.9977 "
18° "	0.9976 "
19° "	0.9975 "
20° "	0.9973 "
21° "	0.9971 "
22° "	0.9969 "
23° "	0.9968 "
24° "	0.9966 "
25° "	0.9963 "
26° "	0.9961 "
27° "	0.9959 "
28° "	0.9956 "
29° "	0.9954 "
30° "	0.9951 "
31° "	0.9949 "
32° "	0.9945 "

The Accuracy of a Quantitative Method. In calculating results of quantitative determinations the accuracy of the result is expressed by the number of figures that are placed to the right of the decimal point. Thus, if we state that the micro-Kjeldahl method showed urine to contain 7.2 g. of total nitrogen per liter we are indicating that the accuracy of the method (or at least the accuracy of our analysis) is not greater than 1 per cent. If we state that by the macro-Kjeldahl method we found the urine to contain 7.19 g. of total nitrogen per liter we are indicating an accuracy of not more than one part in a thousand, or 0.1 per cent.

It is always worthwhile to keep in mind the accuracy obtainable

by a given analytical method and to avoid being over-accurate in measurements that do not require great accuracy, on the one hand, while on the other hand one should be as accurate as possible when making measurements that are vital to precision. A quantitative method is no more accurate than its least accurate measurement. With some methods it is not possible to make determinations much closer than to 2 per cent of the true value. Therefore, it is futile to attempt to obtain an accuracy of, let us say, 0.1 per cent in some other part of the method.

Apparatus to Deliver—Apparatus to Contain. Graduated cylinders and pipettes (usually) are calibrated to deliver specified volumes of liquid and hence actually contain more of the liquid than is expressed in the calibration value, as some of the liquid always sticks to the glass surface.

Volumetric flasks are graduated to contain specified volumes of liquid and hence will never deliver that volume, as some sticks to the glass.

The Conception of an Aliquot. In case one has a solution of certain volume, which contains a precipitate that must be removed before analyzing for a soluble constituent of the liquid, two courses are open:

1. The precipitate can be filtered off, and both filter paper and precipitate can be washed exhaustively to remove all of the solvent clinging to them. The washings are added to the first portion of the filtrate and, after mixing and measuring the total volume, the analysis is made.

2. The volume of the liquid containing the precipitate is determined, if not already known. The material is filtered and a known fraction (*i.e.*, an aliquot) is taken for the analysis. The material which it is desired to analyze can be considered to be evenly distributed throughout the liquid and the precipitate, so that filtering off the precipitate will have no effect upon its concentration. This method is used in blood analysis.

Filtration. When filtering by gravity (using filter paper and filter funnel) the student should keep in mind the following points:

1. The filter paper should be just large enough to come within about 1 cm. of the edge of the funnel. It should not stick above the rim of the funnel, nor should it be of such size as to extend only part way up the side of the funnel. Papers that are too large can be cut down with scissors.

2. The paper should be folded so that it will fit the funnel snugly. When water is not an objection the folded filter can be put in place, filled with distilled water, and tamped down evenly with a clean finger.

3. The filtration funnel should be supported in a shipshape manner by means of a filter arm, or some other suitable contrivance.

4. The receiving vessel should be properly arranged and of the right size.

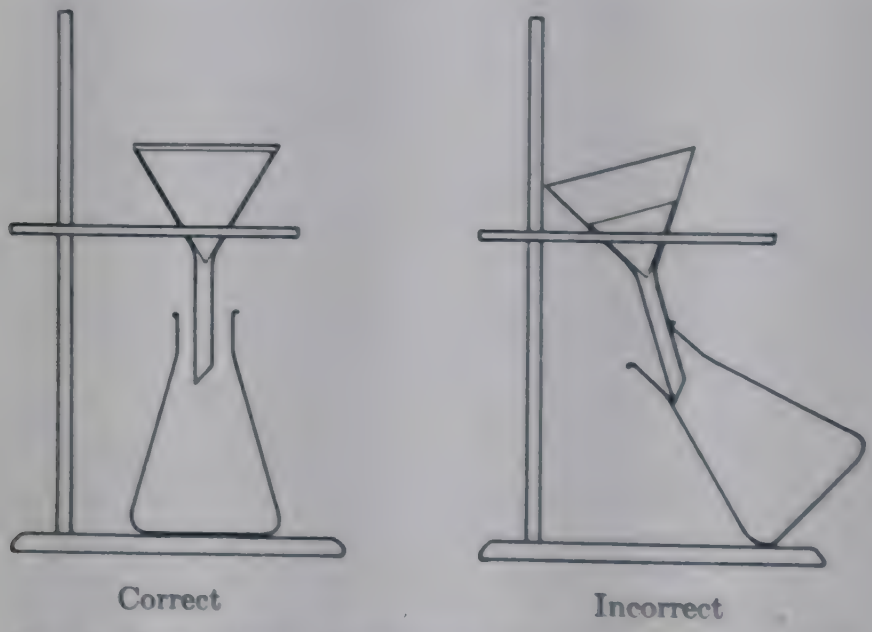


FIG. 1. Filtration. The correct (left) and incorrect (right) way to use a filter funnel.

Contamination of Quantitative Reagents. The student should not stick pipettes wet with water (or other materials) into quantitative reagents. Pipettes must never be placed in the shelf quantitative reagents. Pour the amount you need into a clean dry test tube and pipette from this. Throw away the excess reagent.

The student should not mix up the stoppers of quantitative reagents as the material clinging to the stopper is likely to cause adulteration.

Pinchcocks on Burettes Containing Reagents. When using solutions from burettes the student should take care not to move the pinchcocks from their accustomed positions with the result that the cock does not clamp properly and the liquid is left dripping.

Kjeldahl Flasks. These flasks must be placed in the digestion rack so that they do not touch the back part of the lead pipe. Otherwise ammonium salts may be introduced into the analysis.

Contamination of Rubber Tubing and Stoppers. Rubber tubing and

rubber or cork stoppers that have come in contact with strong acid or alkali must not be used for conveying or for stoppering quantitative solutions of acids or alkalis, as the rubber will have absorbed enough of the strong acid or alkali to affect the value of the quantitative solution.

To Prevent a Quantitative Solution from Running Down the Side of a Beaker When it is Being Poured. Grease the lip of the beaker by rubbing on the nose. Employ a stirring rod to direct the flow of the liquid. It is best to do both.

Parallax. In reading burettes, pipettes, and all graduations on volumetric apparatus, it is necessary to have the eye on a level horizontal with the meniscus. When the eye is below the meniscus the burette reading that appears to correspond with the bottom of the meniscus is below the correct value, and when the eye is above the meniscus the opposite is true. The phenomenon is known as "parallax."

Allowing Burettes to Drain. After running liquid from a burette, a reading should never be made until there has been time for the excess liquid sticking to the walls to drain down. One minute is usually long enough to wait.

Alkali Given off by Glass Containers. Acid solutions that are stored in ordinary glass grow weaker on standing, while alkaline solutions grow stronger. This is because the glass is decomposed and gives off alkali. This can be prevented by drying the glass container, after thorough cleaning, and coating it with paraffin while hot. The excess of paraffin is poured out and the vessel is allowed to cool before use. If the vessel is not perfectly dry and clean when the paraffin is applied, the paraffin will peel off later. It may peel off in any case.

Pyrex glass is useful for the storage of standard solutions of alkali, or of acid, because, unlike ordinary glass, it does not give off alkali.

To determine whether a glass container will or will not give off alkali, fill it with distilled water, add a little phenol red solution, and heat on a steam bath. If alkali is given off by the glass the indicator will turn red. With resistant glass, like pyrex, the indicator will turn orange (owing to the driving off of the carbon dioxide) but will not turn red.

Spilling Nesslerized Solution on the Hands. When using Nesslerized solutions, it is advisable to keep a piece of cheesecloth (to be used for no other purpose) over the left arm. This can be used to catch the drop of Nesslerized solution that starts to run down the edge of the

100 ml. volumetric flask after pouring from it, or the drop that starts down the colorimeter tube after emptying. Otherwise the Nesslerized solution will wet the fingers. This is to be avoided as the solution is both poisonous and corrosive.

The Centrifuge

1. *Balancing the Centrifuge.* When using the centrifuge it is absolutely essential to balance each centrifuge tube and brass holder against an opposing tube and holder. The two sets are placed in balanced cups on the scales, and water is added in sufficient quantity to the balancing tube. If the tubes are not so balanced the centrifuge may be wrecked, or at least damaged.

2. *Rubber Cushions in Tube Holders.* Each brass tube holder has a rubber cushion at the bottom. These cushions must not be lost or misplaced for the tubes will break if centrifuged without them.

3. *Broken Centrifuge Tubes.* If a centrifuge tube breaks while being centrifuged, the centrifuge must be stopped immediately, and every bit of broken glass and other debris must be removed painstakingly both from the centrifuge and from the brass holder. It will be necessary to remove the rubber cushion from the tube holder, to wash it carefully, then dry it and replace it.

4. *Do not Heat Centrifuge Tubes.* Centrifuge tubes that are heated for the purpose of drying them become very brittle and are likely to break while being centrifuged.

5. *Do Not Use Centrifuge Tubes That Are Too Long.* Frequently the centrifuge tube chosen is too long to fit in the holder and will hit the super-structure of the centrifuge when it is extended horizontally by centrifugal force.

6. *Oiling the Centrifuge.* The centrifuge must not be allowed to run out of oil.

7. *Commutator Brushes.* If the centrifuge sparks, or arcs while being run, it is likely that the brushes are worn or not evenly adjusted. It may be necessary to insert new brushes.

Normal Solutions

A normal solution of acid is one which contains 1.0080 g. of ionizable hydrogen per liter. Since the molecular weight of hydrochloric acid is 36.4650 (H = 1.0080, Cl = 35.457) this many grams of hydrogen chloride gas dissolved in water and made up to 1 liter volume will be exactly normal. A $N/10$, or 0.1 N, solution of hydrochloric acid will contain 3.646 g. of hydrochloric acid per liter. The per-

centage of this 0.1 *N* acid will be 0.3646. Ordinary concentrated hydrochloric acid is approximately 42 per cent, or 42 g. per 100 ml., or about 12 *N*.

Sulfuric acid contains two ionizable hydrogen ions; therefore, a normal solution of sulfuric acid will contain in 1 liter the molecular weight in grams divided by 2, or $\frac{98.0760}{2}$, or 49.038 g.

A normal solution of alkali contains 17.0080 g. of hydroxyl ions per liter. A normal solution of sodium hydroxide contains 40.0050 g. per liter (Na = 22.997, H = 1.0080, O = 16.000); a normal solution of barium hydroxide contains 171.376/2 = 85.688 g. per liter (Ba = 137.36, O_2 = 32.000, H_2 = 2.0156), since barium hydroxide contains two ionizable hydroxyl groups.

A normal oxidizing solution contains enough of the oxidizing substance in 1 liter to remove one equivalent of electrons from the material which is oxidized. For example, 1 liter of a 1 *N* oxidizing solution would oxidize 55.85 grams of Fe^{II} to Fe^{III}.

Since 1 mole (the molecular weight in grams) of potassium permanganate can remove five equivalents of electrons, *i.e.*, the manganese is reduced from Mn^{VII} to Mn^{II}, a liter of normal solution of potassium permanganate will contain 158.03/5 = 31.606 g.

One mole of potassium permanganate in acid solution will oxidize 2.5 moles of oxalic acid, $(COOH)_2 \cdot 2H_2O$:

$$2MnO_4^- + 5(COOH)_2 + 6H^+ \rightarrow 10CO_2 + 8H_2O + 2Mn^{++}$$

Therefore, 1/5 mole will oxidize $\frac{2.5}{5}$ or 0.5 mole of oxalic acid and a solution of oxalic acid that is normal with respect to its ability to reduce permanganate will contain per liter 126.068/2 = 63.034 g.

Preparation of Tenth Normal Sodium Hydroxide. One part by weight of sodium hydroxide has been dissolved in one part of water and has been allowed to stand in a large covered cylinder until the sodium carbonate has all settled out. Dilute 9 ml. of this concentrated solution (do not pipette this strong alkali) to 1 liter in a liter volumetric flask. Stopper and mix thoroughly.

Weigh out from a weighing bottle, as accurately as possible, from 0.3 to 0.5 g. of pure benzoic acid, $C_6H_5.COOH$. Place in a 400 ml. beaker. Add 2 or 3 drops of 1 per cent phenolphthalein to 20 ml. of 95 per cent alcohol and add enough of the dilute alkali to produce a barely perceptible pink color. Pour this neutralized alcohol upon the benzoic acid and rotate until it has all dissolved. Now fill a burette

with the diluted alkali (after rinsing twice) and titrate the benzoic acid as carefully as possible. Figure out the normality of your sodium hydroxide solution. The molecular weight of benzoic acid is 122.188. It will not be necessary to make the sodium hydroxide exactly tenth normal.

Note: Benzoic acid has the advantage that it can be readily purified, that it has no water of crystallization, that it is not deliquescent, and that it has a high molecular weight. The higher the molecular weight, the less accurately does the acid used for standardization have to be weighed. Why?

Oxalic acid, $(COOH)_2.2\,H_2O$, can also be used for standardization. For this purpose purest oxalic acid crystals should be powdered and allowed to stand loosely covered with a watch glass on the desk overnight. This is for the purpose of getting rid of included water. The oxalic acid should then be weighed out at once. If allowed to stand over sulfuric acid in a desiccator some of the *water of crystallization* will be lost. Oxalic acid possesses the advantage over benzoic acid of being readily soluble in water, but has the disadvantage of possessing a low molecular weight.

Acid potassium phthalate, $C_6H_4 < \begin{matrix} COOH \\ COOK \end{matrix}$, is still another substance which can be used to standardize alkali solutions. It is easy to purify, has a high molecular weight, and is soluble in water. The purity of a solution can be checked by running a determination of its pH by using the quinhydrone or glass electrode (p. 77). *N*/20 potassium acid phthalate has a pH of 4.00 at 20° C. (MacInnes, D. A., Belcher, D., and Shedlovsky, T., *J. Am. Chem. Soc.* **60**, 1094 [1938]), but since the phthalate is a buffer this pH does not vary much with changes in concentration nor with small amounts of impurities. A 0.05 *N* solution of potassium acid phthalate contains 10.207 g. per liter.

Preparation of Tenth Normal Hydrochloric Acid. Concentrated hydrochloric acid contains about 42 g. of the anhydrous acid in 100 ml., *i.e.*, 42 per cent weight/volume (= wt./vol.). Figure out how much of the concentrated acid will be needed to make 1 liter of tenth normal. Dilute this amount to 1 liter and mix well. Do not pipette the strong acid; measure it with a graduated cylinder. Pipette exactly 25 ml. of the dilute acid into a 250 ml. Erlenmeyer flask and titrate with your standardized alkali. Any of the indicators can be used which change color within the range of pH 4 to pH 10. Why? Calculate the normality of the hydrochloric acid. The molecular weight of hydrochloric acid is 36.46.

Part I

LIPIDS

FATS

1. Solubility. Test the solubility of cottonseed oil in water, cold alcohol, hot alcohol (danger), chloroform, ether (danger), and carbon tetrachloride. Use only 1 ml. of oil for each test. If the oil is soluble, the *clear* solution will give a grease spot when allowed to evaporate upon a piece of paper.

2. Reaction. Test the reaction of fresh, neutral, and rancid cottonseed oil by adding distilled water and a few drops of Congo red and shaking. Congo red is turned blue by fatty acids. Write the reaction, showing the hydrolysis of olein by water.

Neutral oil can be prepared by shaking oil with light magnesium oxide and filtering. It can also be prepared by shaking an ether solution of oil with sodium carbonate solution. The ethereal solution is then separated in a separatory funnel, and the ether is evaporated. The neutral oil becomes rancid quickly if kept at room temperature. However, if one adds a little hydroquinone to it, the oil will stay neutral at room temperature for many months. The hydroquinone acts as an "antioxidant."

3. Acrolein Test. Mix 2 ml. of cottonseed oil with about 2 g. of anhydrous potassium acid sulfate in a dry test tube and heat strongly over a free flame. Smell the fumes of acrolein cautiously. Write the reaction, starting with free glycerol.

4. Emulsification. Shake 2 ml. of neutral cottonseed oil with 5 ml. of water. Shake 2 ml. of neutral cottonseed oil with 5 ml. of dilute sodium carbonate solution. Shake 2 ml. of rancid cottonseed oil with sodium carbonate solution. Shake 2 ml. of neutral cottonseed oil with 5 ml. of egg albumin solution. Emulsions are formed when a hydrophilic colloid is present to exert a protective action upon the droplets formed by shaking. A second function of the colloid is to lower the surface tension and make it easier to break up the oil into small drops. Soap and albumin are hydrophilic colloids.

5. Emulsification without a Protective Colloid. To 10 ml. of distilled water add about 1 ml. of a 10 per cent solution of castor oil in 95 per cent alcohol and mix. Is a permanent emulsion formed?

6. Saponification. Boil 10 g. of fat (preferably bayberry fat) in a large evaporating dish with about 5 ml. of 70 per cent sodium

hydroxide and 300 ml. of distilled water. To test when saponification is complete, see if a few drops of the material dissolve entirely in a test tube of warm distilled water. When saponification is complete, add enough distilled water to the evaporating dish to bring the volume up to about 150 ml. and dissolve all the soap. To one-half of this soap solution add an excess of concentrated hydrochloric acid (approximately 8 ml.). Heat until the melted fatty acids float on top of the solution. Let cool and remove the solid cake of fatty acids. Write the reactions, supposing the fat to be dipalmitylolein. Save the aqueous solution and test it for glycerol. Dissolve about 1 g. of the fatty acids in 4 ml. of alcohol and allow to cool. Observe crystals of impure fatty acid under the microscope by placing a very small drop of the material on a microscope slide. (*Note*: Students must not pour material from a test tube onto a microscope slide and must never use more than a small drop of material, otherwise the microscope will become all wet with chemical solutions.) Is the fatty acid soluble in alcohol? Does it make a grease spot on paper? (*Note*: It may be necessary to melt the fatty acid by heating.)

7. Salting Out. To the other half of the soap solution add solid sodium chloride until no more will dissolve. The soap is salted out. Filter off. Explain "salting out."

8. Insoluble Soaps. Dissolve a little of the soap in distilled water. To a few ml. add a few drops of calcium chloride solution. Repeat, using magnesium sulfate or magnesium chloride solution. Are calcium and magnesium soaps soluble in water? What causes temporary hardness of water? What causes permanent hardness?

9. Reaction of a Double Bond. Add to a solution of cottonseed oil in chloroform a few drops of a dilute solution of bromine in chloroform and shake. Repeat with linseed oil. Repeat with stearic acid. Which of the fatty acids take up the bromine? What products are formed? *Note:* Commercial stearic acid absorbs bromine because it contains small amounts of unsaturated fatty acids. If it is dissolved in chloroform and treated with a slight excess of bromine (in the hood) it will then contain no more unsaturated acids, as these will be completely saturated with bromine.

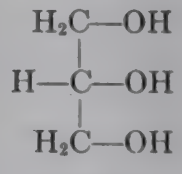
10. Glycerol. What is the taste of glycerol? Is it soluble in ether?

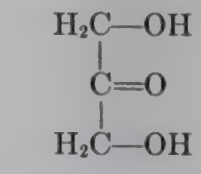
11. Mandel and Neuberg Test for Glycerol (*Biochem. Z.* 71, 214 [1915]). Place one drop of glycerol and 3 ml. of water in a test tube,* add 1 ml. of 0.15 *N* sodium hypochlorite and boil one minute. Add

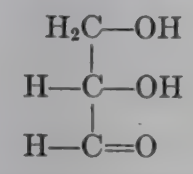
* It is best to perform this test in a large test tube. Add a piece of carborundum to prevent bumping.

3 to 4 drops of concentrated hydrochloric acid and boil one minute to remove the chlorine. Dilute to a volume of 5 ml., add an equal volume of concentrated hydrochloric acid and a *very* small pinch of solid orcinol. Mix and heat to boiling. If directions have been followed, a fine violet color will be produced. If this does not happen, read over the directions again and repeat.

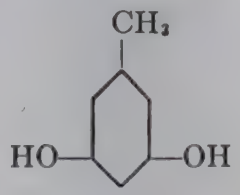
Note: In this test the glycerol is oxidized by the hypochlorite (NaOCl) largely to dihydroxyacetone and partly to glyceric aldehyde:


Glycerol


Dihydroxyacetone


Glyceric aldehyde

These compounds condense with orcinol, when heated with strong hydrochloric acid to form colored compounds. Orcinol, or symmetrical dihydroxytoluene, has the following formula:



The 0.15 *N* hypochlorite solution is prepared by passing chlorine gas into 0.15 *N* sodium hydroxide until a piece of litmus paper is bleached by the product. A suitable solution may also be obtained by diluting commercial solutions of hypochlorite.

12. Copper Complex. To a drop of saturated copper sulfate solution add 5 ml. of water and a drop of saturated sodium hydroxide solution. Mix. What is the effect of adding a drop of glycerol? Is this a good test for glycerol? Compounds with *CH(OH)* groups dissolve metallic hydroxides in certain instances.

13. Lecithin. Mix a little lecithin with water. Is lecithin colloidally soluble in water? Does neutral fat behave in this manner?

14. Lysolecithin (Belfanti, S., Ercoli, A., and Francioli, M., in Bamann-Myrbäck, Die Methoden der Fermentforschung, pp. 88–89 [1940]). Dissect out 15 to 20 stings and poison sacks of dead honey bees and rub them up with 20 g. of egg yolk in a mortar. Mix this material with 500 ml. of *M*/10 phosphate buffer of pH 7.0 to 7.1. Keep overnight at 47 to 48° C. Now test the hemolytic action of the lysolecithin that has been formed by the action of the α-lecithinase. The hemolytic action may be tested by adding 1 to 5 ml. of the lysolecithin suspension to 5 ml. of a beef erythrocyte suspension (1 ml. of

defibrinated beef blood to which has been added 15 ml. of $N/10$ phosphate buffer, pH 7.0 to 7.4). Does hemolysis occur?

Instead of bee stings one can use scorpion stings or snake venom as a source of α-lecithinase. One can also obtain lysolecithin from horse pancreas as follows:

Grind a fresh horse pancreas fine, extract repeatedly with acetone, and then extract the residue 2 or 3 times with 95 per cent ethyl alcohol. The ethyl alcohol extracts are united and evaporated *in vacuo*. Add ether to precipitate the lysolecithin. The precipitate is again dissolved in 95 per cent ethyl alcohol and again precipitated by adding ether.

15. Hydrolysis of Lecithin. Heat some lecithin with concentrated sodium hydroxide in a test tube and observe the odor of trimethylamine. Write the structural formulas of lecithin and trimethylamine.

16. Choline—Rosenheim's Test. Place a *small* drop of choline solution on a microscope slide and add a large drop of Rosenheim's reagent (15 per cent KI solution saturated with I_2). Cover with a cover glass and observe the crystals with the microscope. The compound formed is choline periodide. (*Note:* The crystals will not be obtained unless a large excess of iodine is present). How could one obtain a choline solution, starting with lecithin?

17. Waxes Place 5 g. of spermaceti (cetylpalmitate), 100 ml. of ethyl alcohol and about 1 ml. of concentrated sodium hydroxide in a 500 ml. Erlenmeyer flask. Heat *on the steam bath in the hood* (not at your desks) for 2 or 3 hours, or until all of the ethyl alcohol has evaporated off. Remove a piece of the solid and place it in a test tube. Add distilled water and heat. Note that the soap dissolves, but that the unsaponifiable matter, cetylalcohol, does not. Add 20 ml. of ether (in the hood) to the rest of the solid material in flask. Stir with a glass rod and decant the liquid onto a filter. Allow the filtered ether solution of cetylalcohol to evaporate partially in the hood. Cool and examine for crystals.

18. Detection of Peroxides in Fats and Oils. Dissolve about 1 ml. of fresh cottonseed oil in 2 ml. of chloroform in glacial acetic acid (1 part of chloroform to 2 parts of glacial acetic acid). Add one drop of 10 per cent potassium iodide * solution and shake. Allow to stand for 5 minutes with occasional shaking. Repeat with rancid cottonseed oil, with mineral oil, and with ordinary ether.

* Store in a dark bottle.

What differences in color are noticed? What substance is oxidizing the iodide, and why?

Note: Peroxides represent the first stage in the atmospheric oxidation of fats, waxes, and hydrocarbons. The peroxides gradually decompose, yielding alcohols, aldehydes, ketones, and particularly fatty acids, all of which can be detected by suitable tests. The amount of peroxide present gradually increases, passes through a maximum, and finally falls off, as the oxidation progresses.

19. Fat Unknowns. Apply for two fat unknowns by handing in two clean, dry test tubes bearing your name and consecutively numbered 1 and 2. Each unknown may contain any one or all three of the following: soap, fatty acid, fat. The soap may be an insoluble calcium soap.

You may use the following scheme of analysis: Place about 1 g. of the unknown in a clean, dry test tube. Add 2 ml. of distilled water and 3 drops of phenolphthalein. Heat and shake. If any soap is present, suds will form. The phenolphthalein will be colored pink unless the alkaline reaction due to the soap is masked by the presence of a large amount of free fatty acid.

Now add 5 ml. of 95 per cent ethyl alcohol and titrate carefully with tenth normal sodium ethylate. If free fatty acids are absent, only 2–3 drops of ethylate will be needed to produce a permanent pink. If more ethylate than this is required, continue adding until a faint, but permanent, pink color is obtained. . *Note:* Do not add an excess of alkali. Add just enough to produce a faint pink color. If a large excess of alkali is added, there may not be enough fat present to neutralize this, and the test will always remain pink. (1 ml. of 0.1 *N* sodium ethylate will neutralize the fatty acids set free from 29.3 mg. of olein.) Now stopper the tube *loosely* with a clean cork (to keep out carbon dioxide) and place it in a beaker of water *on* the steam bath (but not *in* the steam bath or the cork will blow out). If a neutral fat is present, it will be seen to settle out at the bottom of the tube in droplets, and after 2 hours or more of heating, the pink color will disappear because the alkali will be used up neutralizing the fatty acids formed by hydrolysis of the fat.

If an insoluble soap is suspected of being present, it can be detected (after making the test for soluble soap and the test for free fatty acids) by washing the material with hot distilled water and then decomposing the insoluble soap by boiling with a little potassium oxalate solution. This will precipitate calcium oxalate and will form a potassium soap, which will form suds.

BRAIN LIPIDS

20. Preparation of Brain Lipids.

Two students are to work together.

Grind 100 g. of calf brain three times in the meat grinder and then extract it for one hour at room temperature with 200 ml. of acetone or 95 per cent ethyl alcohol. Stir from time to time. Filter the material with a fluted Schleicher and Schüll, no. 595, or Whatman, no. 1, filter paper. Allow the brain residue to become partly dry. Then add 200 ml. of ether, transfer to a bottle and let stand over night, tightly stoppered. Leave the material outside on the top of your desk so that if any ether should excape it will not accumulate in one place.

Filter the material the next day. The ether extract contains cholesterol, lecithin, cephalin, sphingomyelin, cerebrosides, and small amounts of other lipids. Add an equal volume of acetone. This precipitates lecithin, cephalin, and cerebrosides. Filter. Cholesterol is in the filtrate.

Wash the acetone precipitate three times with small portions of acetone. Now dissolve the precipitate insofar as possible in about 25 ml. of ether. Let stand for 15 minutes, since the dissolving of lecithin is slow. Filter and wash once with 10 ml. of ether. The filtrate contains lecithin and about 25 per cent of cephalin, together with small amounts of cerebrosides and cholesterol. The residue consists of sphingomyelin and cerebrosides.

Reprecipitate the lecithin and cephalin by adding an equal volume of acetone to the ether solution. The material will separate probably as an oil, which will quickly solidify as the supernatant liquid is poured off, or upon standing in the cold room. In using this crude lecithin for qualitative tests, make a colloidal water solution which should be centrifuged free of any cholesterol which may remain suspended. Lecithin turns brown in the air rather quickly. Why? How could the lecithin be purified further? The best lecithin is no doubt not a chemical individual, but a mixture of lecithins with different fatty acids, and possibly with β-lecithin present. What are the solubility properties of lecithin?

Dissolve the sphingomyelin-cerebroside mixture by adding to it 50 ml. of 95 per cent alcohol and heating on the steam bath. Filter through a warmed funnel. The material will precipitate apparently as "liquid crystals" on cooling. Filter it off and dry it. Test for galactose as follows: To about 0.2 g. add 5 ml. of ethyl alcohol and heat on the steam bath to dissolve. Then add 1 ml. of 3 *N* HCl and

heat on the steam bath for one-half hour. Boil gently to avoid excessive loss of alcohol. Allow to cool and then filter. Add solid sodium acetate to the filtrate until Congo red paper is no longer turned blue. Then add one ml. of phenylhydrazine acetate solution. Heat in boiling water for one half hour, and then allow to cool. Observe the crystals of galactosazone, which have come from the galactose split off from the galactolipids present. It may be necessary to allow the material to stand for some time before the osazone separates out.

Note: The galactosazone may be amorphous. Try crystallizing it from ethyl alcohol if crystals are not obtained at first (see page 58).

To obtain cholesterol, add to the original ether-acetone filtrate, containing the cholesterol fraction, five times its volume of water. An emulsion will form. Now add 1 ml. of concentrated HCl. This will cause the emulsion to "break," and the crude cholesterol will rise to the surface. (If necessary another ml. of concentrated HCl may be added.) Allow the material to stand for one half hour, and then siphon off the liquid below the cholesterol, as far as possible. Filter the cholesterol from the remaining liquid. Allow it to partly dry, and then dissolve it in about 25 ml. of ethyl alcohol, by heating on the steam bath. Filter hot, through a warmed funnel, and allow to cool slowly to room temperature. Large crystals of cholesterol will form. The material should not be allowed to cool below room temperature, or a highly impure product will result. Recrystallize the cholesterol once more and determine the melting point. Test for esters of cholesterol by dissolving about 0.2 g. in hot alcohol and adding phenolphthalein, followed by about three drops of 0.1 *N* sodium ethylate. Let the material stand on the steam bath. If esters are present, the color will gradually fade; but if not, the color will remain. Use the rest of the cholesterol for the qualitative tests.

STEROLS

21. Cholesterol Crystals. Dissolve a minute amount of solid cholesterol in a test tube in a mixture of 5 drops of alcohol and 5 drops of ether. Place a drop of the solution on a microscope slide and examine the crystals under the microscope. Draw a picture.

22. Colors given by Cholesterol Acetate and Cholesterol Benzoate. Heat a mixture of cholesterol acetate and cholesterol benzoate in a test tube until it has just melted. Allow it to cool and observe the beautiful play of colors by reflected light and also by transmitted light.

Note: A tube containing a mixture of cholesterol acetate and cholesterol benzoate may be obtained from the instructor.

23. Lieberman-Burchard Test. In a *dry* test tube place a *small* pinch of cholesterol, 3 ml. of dry chloroform (dried with fused calcium chloride), 1 ml. of acetic anhydride and 1 drop of concentrated sulfuric acid. Mix. Observe the lilac color which soon changes to green.

24. Determination of the Iodine Number of a Fat.

Note: Be sure you are familiar with the directions on pages 5–14 before starting this experiment.

Weigh into a clean, dry, weighed 250 ml. Erlenmeyer flask from 0.1 to 1.0 g. of oil or fat, using less of a highly unsaturated and more of a saturated fat. Add 10 ml. of chloroform and add, from a clean, dry pipette, exactly 25 ml. of Hanus solution. Allow to stand, preferably in the dark, for just 30 minutes, rotating occasionally. Now add 10 ml. of 15 per cent potassium iodide* and mix well. Add 100 ml. of distilled water and titrate to a pale brown color with 0.1 *N* sodium thiosulphate. Now add boiled starch solution and complete the titration. Next, run a blank, using 25 ml. of Hanus solution. Calculate the iodine number of the fat.

Example: 0.20 g. of cottonseed oil required 35.2 ml. of sodium thiosulfate for titration, and 25 ml. of the Hanus reagent in the blank required 57.0 ml. of sodium thiosulfate. The difference

$$57.0 - 35.2 = 21.8 \text{ ml.}$$

of 0.1 *N* iodine used by the oil. The weight of the iodine used is

$$21.8 \times 0.0127 = 0.277 \text{ g.}$$

100 g. of the oil would have used

$$\frac{100}{0.2} \times 0.277 = 138.5 \text{ g. of iodine.}$$

The iodine number of the cottonseed oil is 138.5, since this is the number of grams of iodine used by 100 g. of the oil.

Hanus solution (*Z. Unters. Nahr. Genussm.* **4**, 913 [1901]): Dissolve 13.2 g. of iodine in 800 ml. of pure acetic acid and add to this 3.0 ml. of bromine (danger) dissolved in 200 ml. of acetic acid. Mix well.

25. Determination of the Thiocyanogen Number of a Fat. (Martin and Stillman modification of Kaufman Volumetric Method, *Oil and Soap* **10**, 29 [1933]).

**Note:* This potassium iodide must be kept in a brown bottle, or else it will decompose and liberate iodine.

Weigh 0.1 to 0.2 g. of the fat or oil (excess of the thiocyanogen reagent should be 150 to 200 per cent) into a 200 ml. glass-stoppered bottle or flask. Add 25 ml. of the thiocyanogen solution from a pipette, rotate the bottle until the fat is dissolved, and allow it to stand in the dark for 20 to 24 hours. Add 10 ml. of 10 per cent potassium iodide solution quickly and at one time, while shaking the bottle to avoid hydrolysis of the thiocyanogen solution. Add 100 ml. of water and titrate the liberated iodine with standardized 0.1 *N* thiosulfate solution in the usual manner, using starch indicator. Conduct at least two blank determinations along with the determination on the sample. Subtract the number of ml. of thiosulfate solution required by the sample from the number required by the blank. Multiply this difference by the iodine equivalent of the thiosulfate solution. The value obtained is the quantity of iodine equivalent to the thiocyanogen absorbed by the fat or oil. Calculate the percentage by weight and report as thiocyanogen number.

To prepare the thiocyanogen solution one needs (a) lead thiocyanate, (b) anhydrous acetic acid, (c) a solution of bromine in carbon tetrachloride and acetic acid, (d) specially purified carbon tetrachloride, and (e) a solution of lead thiocyanate in anhydrous acetic acid. These are prepared as follows:

a) Lead Thiocyanate. Dissolve 331 g. of pure lead nitrate in 700 ml. of water and filter. Dissolve 194 g. of pure potassium sulfocyanate in 500 ml. of water and filter. Slowly add the lead nitrate solution to the potassium sulfocyanate solution with stirring. Continue stirring for 30 minutes and allow the precipitate to settle. Decant the supernatant liquid through filter paper on a Büchner funnel, using slight suction, and wash the precipitate several times with water by decantation. Transfer the precipitate to a Büchner funnel, using a horn spoon and water, and wash with water until the washings give no test for nitrate. Place the precipitate on a watch glass and dry it to constant weight (*ca.* 7 days) in a vacuum desiccator over sulfuric acid. The lead thiocyanate should be white in color. Store it in an air tight brown bottle and keep it in the dark (yield *ca.* 260 g.).

b) Anhydrous Acetic Acid. Boil gently in a liter flask with ground-in glass air condenser for approximately 1 hour, 500 ml. of acetic acid containing at least 99.5 per cent acetic acid, with 40 ml. of acetic anhydride. Attach a calcium chloride tube to the end of the condenser and allow the acid to cool to room temperature.

c) Bromine Solution. Weigh 4.2 g. of dry bromine into a 250 ml. volumetric flask. Dissolve it in 100 ml. of the pure dry carbon tetra-

chloride and fill to the mark with anhydrous acetic acid.

d) Carbon Tetrachloride is shaken with pure, concentrated sulfuric acid (50 ml. per liter of CCl_4) in a separatory funnel and is allowed to stand for 2 hours. The acid is drawn off and the treatment is repeated with fresh acid until, after standing for 2 hours, the color of the acid layer is no darker than a light straw color. After separating the acid, the carbon tetrachloride is washed with water to remove most of the acid. It is then washed twice with 50 per cent KOH (50 ml. per liter of CCl_4). Most of the remaining water is removed by allowing the carbon tetrachloride to stand for several hours over pellets of KOH. The CCl_4 is then distilled. The last traces of moisture are removed by drying over phosphoric anhydride (50 g. per liter). Filter the CCl_4 into a distilling flask that contains 10 g. of P_2O_5 for each liter of filtrate. Distill in an all-glass still. All equipment used in distilling the dry CCl_4 must be dried for at least 1 hour at 120° C. The use of a two- or three-necked receiving flask is recommended. One outlet should be fitted with a drying tube (*Ind. Eng. Chem. Anal. Ed.* **17**, 336 [1945]).

e) Lead Thiocyanate Solution. Pour 250 ml. of anhydrous acetic acid on 12.5 g. of the pure dry lead thiocyanate in a white, dry, glass-stoppered liter bottle.

To prepare the *thiocyanogen solution* add the bromine solution to the lead thiocyanate solution in small quantities. Give the latter a vigorous shaking after each addition and take care that decoloration takes place before each addition of the bromine solution. After the two solutions are completely mixed, allow the suspension, consisting of precipitated lead bromide and excess lead thiocyanate, to settle. Filter the solution through dry paper into a dry, brown, glass-stoppered bottle. Keep the filtrate, which should be clear and colorless, or only slightly yellow, in the dark. The solution, if correctly prepared, will require 24–26 ml. of 0.1 *N* thiosulfate solution for its iodometric titration. The thiocyanogen solution will keep approximately 1 week. After that time it begins to show a yellow color and a turbidity. Soon a fine yellow precipitate settles to the bottom of the bottle.

BILE

26. Shake a little bile with 10 ml. of water. Observe the color and the production of foam.

27. Bile Pigments: Gmelin's Test. Place about 3 ml. of bile in a test tube and carefully underlay with about 1 ml. of yellow concentrated nitric acid. This can be done easily if one places a filtration

funnel (with a stem long enough to reach the bottom) in the test tube, and pours the nitric acid down the funnel. Observe the various colors. Bile contains the two pigments bilirubin, which is yellow, and biliverdin, which is green. The nitric acid oxidizes the pigments to form various products, among which are: biliverdin, bilicyanin and cholетelin.

Various other oxidizing agents can be used instead of nitric acid. Try Hammarsten reagent. This is prepared by adding 17 ml. of concentrated nitric acid to 50 ml. of concentrated hydrochloric acid (in the hood) and, after the mixture has just turned yellow, adding 300 ml. of ethyl alcohol.

28. Bilirubin. To about 2 ml. of bilirubin solution add 2 ml. of 0.4 *M* phosphate buffer of pH 7.4. Now add 10 to 20 drops of freshly mixed Ehrlich reagent. If bilirubin is present a red color will appear. Biliverdin will give no color.

The Ehrlich reagent is prepared as follows: *Solution* I: Dissolve 5 g. of sulfanilic acid and 50 ml. of concentrated HCl in water, dilute to 1 liter and mix. *Solution* II: Dissolve 5 g. of sodium nitrite in water and dilute to 1 liter. For use mix 33 ml. of solution 1 with 1 ml. of solution II.

29. Bilirubin to Biliverdin: To a solution of bilirubin add a drop of peroxidase and a drop of 3 per cent hydrogen peroxide. Biliverdin is formed.

30. Mylius-Pettenkofer Test for Bile Acids. Place 5 drops of bile and 3 ml. of water in a test tube. Add 5 drops of a 1–1000 furfural solution and mix. Pour 1 ml. of concentrated sulfuric acid down the inclined test tube. Observe the pink ring. The test is due to the presence of glycocholic and taurocholic acids.

31. Hay Test for Bile Acids. To 5 ml. of water in a test tube add 1 ml. of bile and mix. Drop into this a pinch of finely powdered sulfur. What happens to the sulfur? Repeat, using distilled water and no bile. Bile acids greatly lower the surface tension of water. Explain how lowering of the surface tension makes easy the formation of foam.

ACETONE BODIES

32. Acetone: Lieben Test. To 5 ml. of acidosis urine add about 10 drops of saturated sodium hydroxide and mix Filter off the precipitated tri-calcium phosphate. To the clear filtrate add 3–4 ml. of iodine solution (iodine dissolved in aqueous potassium iodide and known as potassium tri-iodide or as Lugol's iodine solution). Mix

and observe the crystals of iodoform under the microscope. Observe the odor also.

33. Acetone: Gunning Test. To 5 ml. of acidosis urine add 3 ml. of ammonium hydroxide, mix and filter. Now add 3–4 ml. of iodine solution, mix, and let stand 10 minutes, or if necessary, for several hours. If traces of acetone are present the separation of iodoform will not occur for a considerable period of time. This test is negative in the presence of ethyl alcohol and acetaldehyde.

The chemical reactions involved in Exp. 32 are as follows:

$$I_2 + 2\,NaOH = NaOI + NaI + H_2O$$

The sodium hypoiodite, NaOI, slowly changes to sodium iodate, $NaIO_3$, which is not concerned in the formation of iodoform:

$$3\,NaOI = NaIO_3 + 2\,NaI$$

With sodium hypoiodite acetone first reacts to form tri-iodo acetone:

$$3\,NaOI + \begin{matrix} CH_3 \\ | \\ C{=}O \\ | \\ CH_3 \end{matrix} = \begin{matrix} CH_3 \\ | \\ C{=}O \\ | \\ CI_3 \end{matrix} + 3\,NaOH$$

Tri-iodo acetone

In the presence of sodium hydroxide, iodoform and sodium acetate are formed:

$$\begin{matrix} CH_3 \\ | \\ C{=}O \\ | \\ CI_3 \end{matrix} + NaOH = \underset{\text{Iodoform}}{CHI_3} + \underset{\text{Sodium acetate}}{CH_3{-}CO{\cdot}ONa}$$

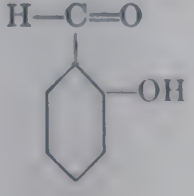
In Exp. 33 the iodine first reacts with the ammonia to form nitrogen tri-iodide.

$$2\,NH_4OH + 6\,I_2 = 2\,NI_3 + 2\,H_2O + 6\,HI$$

Then this forms *in statu nascendi* tri-iodo acetone.

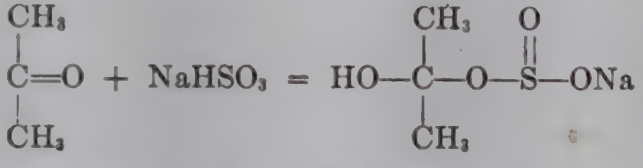
34. Acetone: Salicylic Aldehyde Test. To 5 ml. of acidosis urine add 3 drops of a 10 per cent solution of salicylic aldehyde in absolute alcohol and mix. Pour down the side of the test tube 1–2 ml. of concentrated sodium hydroxide and without mixing allow the tube to stand for 5 minutes. If acetone is present a deep orange color will appear. In the absence of acetone the color will be yellow.

The structural formula for salicylic aldehyde is:

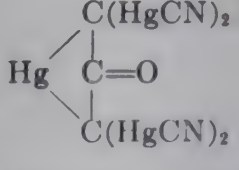


35. Acetone by Aeration Given as a Demonstration). When tests for acetone are made with urine it is always possible (though not usual) that the urine may contain some interfering substance such as alcohol, aldehyde, or protein. It is sometimes advisable to distill off the acetone, or still better, to remove it by a strong stream of air (aeration).

Place 5 ml. of acidosis urine and 1 ml. of 10 per cent sulfuric acid in a large test tube. In another large test tube place 10 ml. of fresh 2 per cent sodium bisulfite solution. Connect up with the aeration apparatus to aspirate the acetone from the urine into the tube containing the bisulfite. A moderate air current should be employed, and the urine tube should be placed in a beaker of water at 30° to 40° C. The acetone removed by the air current is taken out by the bisulfite, the reaction being:

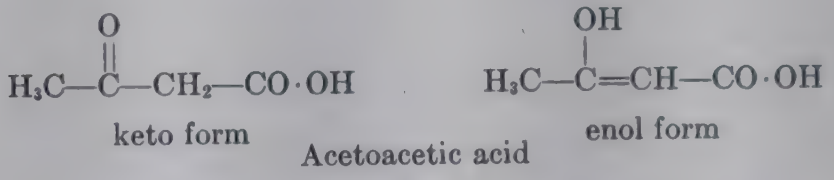


The acetone-bisulfite can be used as in the usual tests for acetone, or a quantitative method can be used. One of the most delicate of the tests is that performed with the Scott-Wilson reagent. Add a few ml. of freshly prepared Scott-Wilson reagent to a little of the acetone-bisulfite and observe the colloidal suspension that is formed. This is the basis of a quantitative nephelometric method for the determination of acetone. This reagent soon deteriorates. The precipitate formed by adding the reagent to acetone is (Kline, L., *Biochem. Z.* **273,** 1 [1934]):



Preparation of Scott-Wilson reagent: Folin, O., *J. Biol. Chem.* **18,** 265 (1914). To 10 g. of mercuric cyanide dissolved in 600 ml. of water add a cooled solution of 180 g. of sodium hydroxide in 600 ml. of water. Add 2.9 g. of silver nitrate dissolved in 400 ml. of water. Add the silver nitrate solution slowly and stir the solution vigorously during the addition. If the solution is turbid, it should be set aside to settle for three or four days and the clear supernatant liquid removed by means of a siphon.

36. Acetoacetic Acid: Gerhardt Test. To about 5 ml. of *fresh* acidosis urine add 10 to 15 drops of 10 per cent ferric chloride. Notice the port wine color. This is due to the formation of a ferric compound with the enol form of acetoacetic acid.

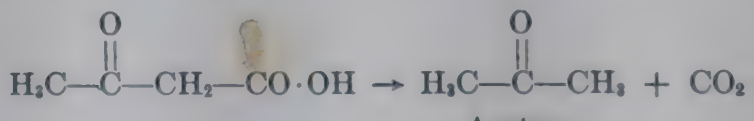


Acetoacetic acid

Heat to boiling and note that the color disappears, since the heat decomposes the acetoacetic acid to acetone and carbon dioxide. Repeat the test, this time with normal urine. In this case there is no production of a port wine color and the only result is the precipitation of ferric phosphate. The Gerhardt test is not especially sensitive. It is simulated by salicylic acid and compounds of salicylic acid, which, when given as medicine, reappear in the urine.

Test some normal urine to which a small pinch of salicylic acid has been added. Note that the color is more purplish than that given by acetoacetic acid and that it does not disappear on boiling, inasmuch as the salicylic acid is stable.

Note: If acidosis urine is not available, one can employ normal urine to which acetoacetic acid has been added. The acetoacetic acid can be prepared by allowing a 1 per cent solution of ethyl acetoacetate to stand over night with a slight excess of sodium hydroxide. This hydrolyzes the ester and liberates free acetoacetic acid. Acetoacetic acid is very unstable and soon decomposes as follows:



Acetone

Ethyl acetoacetate itself gives the Gerhardt test, but gives a brown color in the Le Nobel test instead of a purple.

37. Acetoacetic Acid: Le Nobel Test. To 5 ml. of acidosis urine add a few drops of freshly prepared 5 per cent sodium nitroprusside solution and 2 ml. of 10 per cent acetic acid. Mix well. Pour on the surface of the liquid an excess of ammonium hydroxide. This will be facilitated if the test is carried out in a narrow tube. If acetoacetic acid is present, even in traces, a purple ring will be formed. Acetone also gives this test, but only if present in considerable concentration.

The reaction described above is given with compounds containing

the "SH" group, *i.e.* sodium hydrosulfide, reduced glutathione, etc.

FORMALDEHYDE AND METHYL ALCOHOL

38. Formaldehyde: Orcinol Test. To 2 ml. of the aqueous solution suspected of containing formaldehyde add 10 drops of the modified Bial's reagent (see Exp. 58) and 2 ml. of concentrated hydrochloric acid. If the original liquid contained as much as 1 mg. of formaldehyde per ml. an immediate white precipitate will form. If no precipitate forms on standing, heat the tube in boiling water for 15 minutes. If traces of formaldehyde are present, the liquid will assume an orange color. Cool the tube in running water, dilute with 5 ml. of distilled water, and cautiously add an excess of concentrated sodium hydroxide. If as much as 1 part of formaldehyde was present in 1,000,000 parts of the original solution, a greenish fluorescence will be seen on looking down through the liquid against a black background. The fluorescent compound is presumably a diphenyl methane coloring matter. With formaldehyde solutions containing more than 0.01 mg. per ml. the fluorescent compound is not formed.

Note: If you get a precipitate in the first part of this test, the test is positive, and it is useless to proceed further. If you wish to observe the greenish fluorescence, dilute the formaldehyde solution until you can no longer get the precipitate. Then heat in boiling water, cool, dilute, and add the sodium hydroxide.

39. Methanol (Methyl Alcohol): Orcinol Test (Sumner, J. B., *J. Am. Chem. Soc.* **45**, 2378 [1923]). Place in a large test tube which is 25 mm. in diameter and free from scratches, 1 ml. of the suspected alcohol, 2 ml. of 6.7 per cent potassium dichromate solution, and 2 ml. of 1 to 2 (67 per cent) sulfuric acid. Mix at once after adding the sulfuric acid, and allow to stand at room temperature for about 10 minutes. The reduction of the chromic acid to blue chromous sulfate should take about 40 seconds, and if it takes much longer than this, the alcohol used contains too much water. Add 15 ml. of distilled water, mix thoroughly, and heat in boiling water for 10 minutes. Now add 5 mg. of orcinol in 1 ml. of water, mix very thoroughly, and heat in boiling water for 30 minutes. If the alcohol used contained 5 per cent or more of methyl alcohol, a precipitate will form after about 5 minutes heating. With 1 per cent of methyl alcohol, a precipitate will form after 15 minutes heating. This test will show methyl alcohol down to 0.5 per cent, although in this case it may be necessary to heat

for 30 minutes, and then allow the solution to cool before a precipitate forms. If the precipitate is filtered off, it will be seen to be distinctly brown or yellow.

Quantities of methyl alcohol smaller than 0.5 per cent can be detected by precipitating the chromium by adding a slight excess of sodium hydroxide and heating. When this precipitate is filtered off, the clear filtrate possesses a green fluorescence if even traces of methyl alcohol were originally present. This last procedure is of doubtful value, as it is to be expected that traces of methyl alcohol derived from pectinous substances may possibly be present in beverages from fruits.

The alcohol used in the test is obtained by distilling the suspected solution or beverage, using a Vigreux column, or a still more efficient one, to obtain as complete a separation from water as possible. The temperature in the upper portion of the column should not be allowed to exceed 80° C.

40. Dialysis. Cut off a strip of cellophane tubing of the required

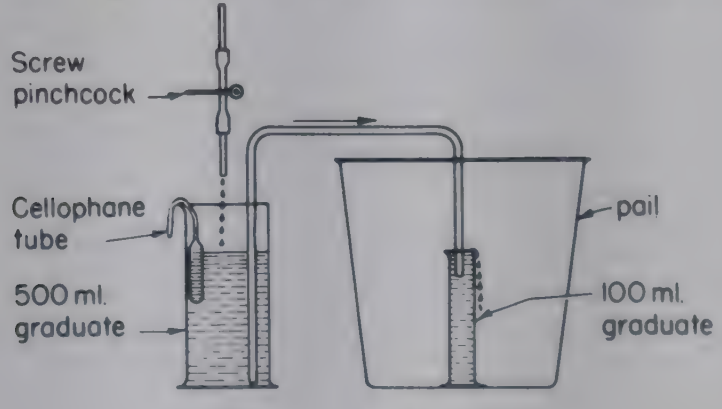


Fig. 2. Dialysis.

length and wet it well with distilled water. Now tie a firm knot in one end. Open the other end of the tubing and fill it with distilled water to test for leaks. If there are no leaks, pour out the water and fill the tubing (never more than one-half full) with the solution which is to be dialyzed. Suspend the tubing in a 500 ml. graduate and press the empty end of the tubing down over the outside of the graduate. The graduate should be one-half to two-thirds full of water. It may be advisable to add toluene to the dialyzing material. It is usually advisable to keep dialyzing preparations in the ice chest.

When one desires to dialyze against running distilled water the setup shown in Fig. 2 can be employed:

CARBOHYDRATES

41. D-Glucose. Observe the appearance of crystals of pure *d*-glucose both macroscopically and under the microscope. Observe also granules of commercial glucose.

42. Molisch-Udransky Test. Place 4 ml. of 0.5 per cent glucose solution in a test tube, add 2 drops of Molisch reagent (10 per cent α-naphthol in alcohol), and mix. Pour down the inclined test tube about 2 ml. of concentrated sulfuric acid. Agitate very slightly. A purple ring will form. Repeat, using a 0.01 per cent glucose solution. This test is given by all carbohydrates and by all compounds containing carbohydrate groups. The carbohydrates form furfurals: the aldopentoses and pentosans are converted to furfural, while the ketohexoses, aldo-hexoses, and polysaccharides built of these units form hydroxylmethylfurfural, when acted on by the sulfuric acid, and these condense with the α-naphthol to produce colored compounds.

In running qualitative tests like the Molisch-Udransky on an unknown solution, it is sometimes advisable to carry out a blank (using distilled water instead of the sugar solution) as well as a control (using 4 ml. of a solution containing, let us say, 0.02 mg. of glucose per ml.). The blank must give no violet color; the control must give a perceptible violet color.

43. Phenylhydrazine Test. Place 5 ml. of 0.5 per cent glucose solution * in a test tube and add 1 ml. of the phenylhydrazine acetate solution. Mix. Stopper with a wad of cotton and heat in boiling water for about 20 minutes. Place a *small* drop of the material on a microscopic slide and observe crystals under the microscope. Glucosazone is insoluble in hot water. (The osazones of some sugars are soluble in hot water and precipitate only upon cooling.) Write the reactions for the formation of glucosazone.

The phenylhydrazine solution is made by adding 10 ml. of glacial acetic acid to 20 ml. of phenylhydrazine and 200 ml. of water. As a preservative add also 2 g. of sodium bisulfite. Filter through cotton until clear, if necessary. *Do not inhale vapors of phenylhydrazine or allow the material to get on the fingers, as it is very poisonous.*

The phenylhydrazine test is capable of detecting glucose in pure

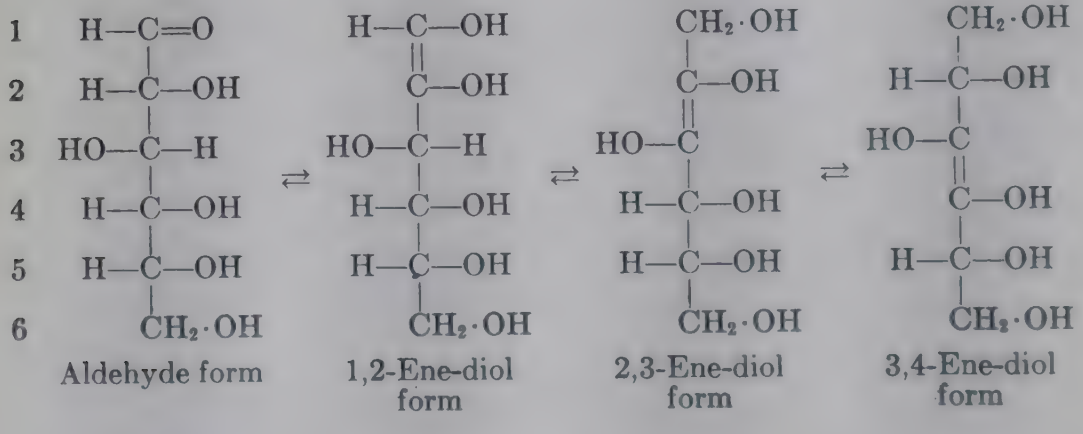
* Solutions of the various sugars, except sucrose, can be conveniently prepared using a saturated aqueous solution of benzoic acid as a solvent. Such solutions keep indefinitely.

solution when only 0.01 per cent is present.

44. Diffusibility of Glucose. Make a concentrated solution of glucose by mixing a teaspoonful with 3 ml. of water. Place it within a collodion sack (see Exp. 40). Observe the diffusion streams descending from the bottom of the sack. After one-half hour mix the outer liquid and test for glucose. Glucose is a crystalloid and diffuses readily. Place some Congo red within the sack. Congo red gives a colloidal solution. In this way the sack can be shown to contain no holes.

45. The Enolization of Glucose. Boil 2 ml. of glucose solution with exactly 5 ml. of 0.1 *N* sodium hydroxide and observe the production of caramel. (This is *Moore's test.*) Now test for the presence in the solution of enol forms by adding to the cooled solution exactly 5 ml. of 0.1 *N* hydrochloric acid containing 1 ml. of 10 per cent ferric chloride. If enolized glucose is present a transitory plum color will be observed. Ordinary glucose does not give this color.

The *formation of enol forms of glucose* is believed to be as follows:



46. Barföd's Test. Boil 5 ml. of Barföd's reagent with 1 ml. of glucose solution for 10 seconds. Cuprous oxide is precipitated. This test is used to distinguish between the monosaccharides and such disaccharides as maltose and lactose, which do not reduce Barföd's reagent except after longer boiling. When only a trace of monosaccharide is present, a slight precipitate of cuprous oxide will be seen to settle out on the bottom of the test tube upon allowing the tube to stand for 4 or 5 minutes after heating. Concentrated solutions of maltose, or lactose, will give slight reductions with Barföd's reagent on long boiling.

Barföd's reagent (modification by Tauber, H., and Kleiner, I. S.,

J. Biol. Chem. **99**, 249 [1932]) is prepared by dissolving 24 g. of cupric acetate in 450 ml. of boiling water and adding at once 25 ml. of 8.5 per cent lactic acid. The material is stirred, cooled, diluted to 500 ml. and filtered.

Note: Barföd's reagent can not be used with sugar solutions containing chlorides. In this case a white precipitate of basic cupric chloride is formed upon heating.

47. Benedict's Test (Benedict, S. R., *J. Biol. Chem.* **3**, 101 [1907]; **5**, 485 [1909]). Boil 8 drops of *sugar* solution, or of urine, with 5 ml. of Benedict's solution for exactly 2 minutes. Do not boil so vigorously that much of the solution is boiled away. It is permissible to heat the test tube in boiling water; the time should be just 3 minutes in this case. Observe the precipitate. If (with urine) the test is *turbid*, the urine can be said to contain pathological amounts of sugar. When used with normal urine, no precipitate, other than a slight haze of copper urate, should be seen.

The students are given several samples of urine containing unknown concentrations of glucose and are asked to compare the reductions of this urine with the reductions given by solutions of known glucose content.

In this reduction test, the copper, Cu^{II}, is reduced to cuprous oxide, Cu^{I}_2O. The glucose is oxidized. Possibly gluconic acid is the first oxidation product, but in hot alkaline reagents such as this a number of other products are formed rapidly (see Raymond, A. L., in Gilman, H., Organic Chemistry, John Wiley and Sons, pp. 1519–1525 [1938]).

Benedict's reagent is composed of copper sulfate, sodium carbonate, and sodium citrate in one solution. It keeps perfectly and is an extremely reliable reagent for the examination of urine for pathological amounts of sugar.

To prepare the reagent dissolve 173 g. of sodium citrate (Na_3 Citrate · $2H_2O$) and 100 g. of sodium carbonate, *anhydrous*, in 800 ml. of warm water. Filter and dilute to a volume of 850 ml. Dissolve 17.3 g. of pure crystallized copper sulfate in 100 ml. of water and add slowly and with stirring to the first solution. Dilute to 1000 ml.

48. Picric Acid Test. Heat 1 ml. of glucose solution with 5 ml. of a saturated picric acid solution and 1 ml. of 20 per cent sodium carbonate solution. Picramic acid is formed by reduction of one nitro group, ortho to the hydroxyl. Write the reaction. This is the basis of the Lewis-Benedict method for blood sugar. The Benedict-Oster-

berg method for sugar in urine is similar, but requires acetone because the picric acid would otherwise react with the creatinine in the urine.

49. The Use of a Photoelectric Colorimeter. In recent years photoelectric colorimeters have largely replaced the previous type, such as the Duboscq, which depend upon a visual matching of the unknown with a standard. Some principles involved in the use of photoelectric colorimeters are detailed below. Briefly, the use of such colorimeters depends upon graphs which are constructed for each analytical method. Each such graph can be used only with the particular instrument for which it was prepared. It cannot be used even with another instrument of the same make. The graph is prepared by running the colorimetric method in question, using accurately prepared standards of suitable dilutions. The readings thus obtained are plotted on coordinate paper against the concentration of the substance in question. The colorimeter is adjusted to the proper reading (either the null point or 100 per cent transmission, depending upon the type of instrument) by using a blank solution. Then the unknown solution is substituted for the blank and the colorimeter reading again obtained. The amount of material corresponding to this reading is found by consulting the graph. The student must note what final dilution has been used in constructing the graph. Thus a graph may have been prepared by reading solutions containing ammonia nitrogen Nesslerized and diluted to 200 ml. If the student has diluted his unknown to 50 ml. he must divide his value by 4 to obtain the correct results.

At present there is a variety of photoelectric colorimeters available. In general these may be divided into two types. One type balances one photocell against another similar photocell, in much the same as one half-cell is balanced against the other in the measurement of pH with a potentiometer.

One photocell receives light that has passed through the solution to be measured, and the other receives light directly from the light source. In the second type of instrument there is only one photocell. The amount of current produced by this photocell is measured directly by means of a suitable instrument, *e.g.*, a microammeter. Various kinds of photocells may be used. The two common types in use are the barrier type cells and vacuum phototubes.

The two types of photoelectric colorimeters will be described briefly.

One-photocell type: Fig. 3 illustrates a simple photoelectric colorimeter using a single barrier-type photocell. With suitable modifica-

tion this arrangement could use any photocell, or phototube.

In the use of this type of colorimeter, tube No. 1 is filled with the reference solution. This is usually the solvent in which the unknown is dissolved, or is distilled water. Tube No. 1 is placed in front of the photocell and the light intensity is adjusted by means of the shutter until the microammeter reads some convenient value, *e.g.*, 100. This reading will be called I_s. The unknown, in tube No. 2, is then placed in front of the photocell and the reading is again taken, without changing the shutter. This reading will be called I. The I_s reading should again be checked to make sure there has been no change in the light intensity. If I_s has changed, the reading for I must be repeated. This must be repeated until consistent results are obtained.

From the ratio I/I_s one can calculate the concentration of the unknown solution, as will be shown below.

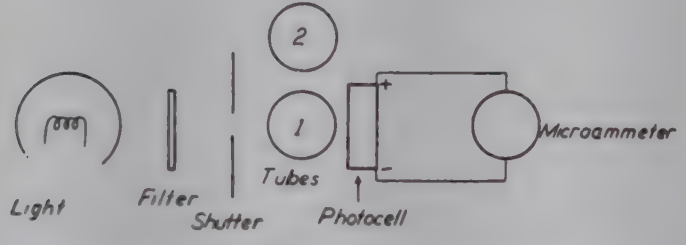


FIG. 3. Diagram of a photoelectric colorimeter using one photocell.

The filter (see Fig. 3) is used to transmit light of more or less restricted wave lengths. In the more elaborate spectrophotometers, monochromatic light is provided by means of diffraction gratings and/or prisms.

Instead of a shutter to control the light intensity, one might use various other means. Some of these are, (1) a neutral wedge, *i.e.*, a wedge-shaped piece of glass which is of neutral tint; (2) a rotating sector, *i.e.*, a rotating disc in which a variable portion is cut away to allow the light to pass; (3) by using two Nicol prisms, much as the polarizer and analyzer in the polarimeter.

Two-photocell type: In Fig. 4 is illustrated a convenient type of colorimeter using two barrier-type photocells. With suitable modifications, vacuum phototubes could be used also. In this figure, R_1 and R_2 are variable resistances. R_1 is a graduated, uniform slide wire. It may be graduated in uniform divisions or may be graduated with a logarithmic scale. A single light source is used and by means of a mirror, or some other arrangement, both photocells are illuminated

by the single light source.

In using this type of instrument, tube No. 1, which contains the reference liquid, is placed before photocell II. With R_1 set at the desired value (100 if uniformly graduated, or at 0 if the scale is 100 log I_s/I), adjust R_2 until there is no deflection of galvanometer, G. This reading of R_1 is called I_s.

Now move the unknown solution in tube No. 2 before photocell II and adjust R_1 until there is again no deflection of the galvanometer.

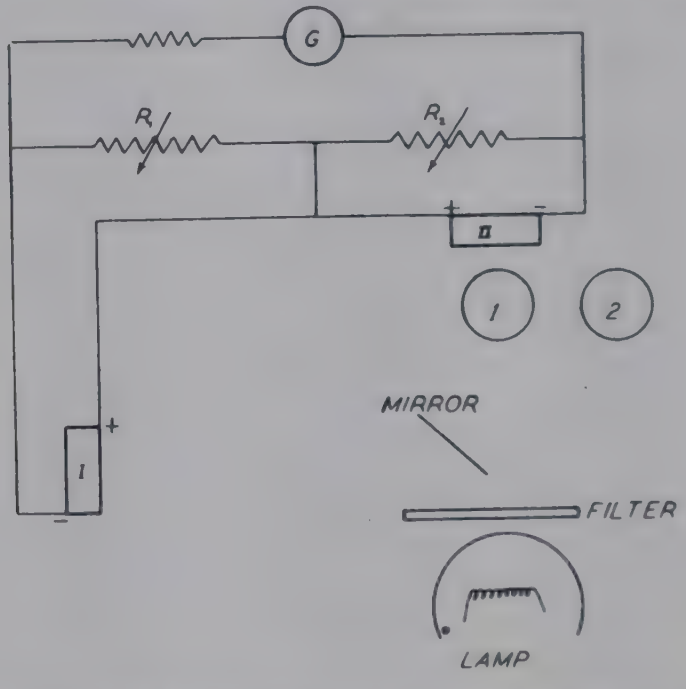


FIG. 4. Diagram of a photoelectric colorimeter using two photocells.

The reading of R_1 is now I. From the ratio I/I_s one can calculate the concentration of the unknown.

Various other arrangements have been made using two photocells, but the general principles are the same. In any case the specific directions for any particular apparatus must be studied before it is used.

To calculate the concentration of the unknown solution one must first obtain a calibration curve. This is done by using various amounts of the material to be measured, *e.g.*, various concentrations of KH_2PO_4 if one wishes to calibrate a curve for phosphorus determinations. According to Beer's law:

$$I = I_0 10^{-Ecl}$$

Where E is the extinction coefficient, c is the concentration and l is the length of solution through which the light passes. I is the intensity

of the light after passing through the solution and I_0 is its intensity before entering the solution. In practice in colorimetry, I_0 is replaced by I_s, which is the light intensity of the light after having passed through the solvent in which the colored material is to be dissolved. This substitution eliminates the rather difficult measurement of I_0. (I_s is frequently written as I_0. This is not correct, but it causes little confusion because its meaning is generally understood.)

The above equation may be written:

$$\log \frac{I}{I_s} = - Ecl$$

From this it is apparent that if $\log \frac{I}{I_s}$ is plotted against concentration, for any given tube, a straight line will be obtained, the slope of which will be $- El$. Frequently absorption cells with parallel sides are used instead of the round tubes illustrated in Figs. 3 and 4. In such a case l can be measured readily, and E can be determined. With round tubes, the evaluation of l is very difficult and is seldom attempted.

Rather than calculating $\log I/I_s$ for each reading, one can plot the calibration curve on semi-logarithmic graph paper. The ratio I/I_s represents the fraction of light transmitted through the colored solution. If I_s is always set at 100, this ratio can be read directly in per cent of light transmitted. The fraction of light transmitted is plotted on the logarithmic scale, and the concentration is plotted on the uniform scale of the graph paper. If the solution obeys Beer's law, the result should be a straight line. Frequently the result is not quite a straight line.

Some commercial types of photoelectric colorimeters have logarithmic scales so that $\log I/I_s$, or some similar relation, *e.g.*, $100 \log I_s/I$ is read directly. In this case the scale readings can be plotted against concentration on ordinary graph paper. The result should be a straight line if Beer's law applies.

Once a calibration curve has been obtained, the concentration of an unknown solution can be determined readily by reference to this curve.

Precautions:

1. Be sure you are familiar with the directions for using the particular apparatus you are to use.

2. Remember that this is an expensive apparatus.

3. Be sure the tubes, or absorption cells, are clean. They must be clean and dry on the outside. The inside must also be clean and dry, or else it must be rinsed thoroughly with the solution to be measured. Avoid scratching the surfaces of the tubes.

4. Be sure the tube used for reference is comparable with the tube used for the unknown. To do this, fill both tubes with the same solution and see if the same reading is obtained with each of them.

5. Be sure you have the proper light filter.

6. If round tubes are used, care must be taken to always place them in the holders in the same way, unless you are sure the tubes are of uniform thickness all the way around and are perfectly round, which is seldom true. Also, with round tubes, it is very important that the tube holder is in the same position each time readings are made. A slight change in position may influence the reading.

7. Always leave the tubes clean when you have finished.

8. Always turn off the instrument when you have finished, unless you are sure someone else is going to use it immediately.

50. Dinitrosalicylic Method for Glucose (Sumner, J. B., *J. Biol. Chem.* **65,** 393 [1925]). With a Folin-Ostwald pipette (see page 6) place exactly 1 ml. of the diluted diabetic urine, or of glucose solution, containing 0.2 to 2.0 mg. of glucose, in a clean Folin-Wu sugar tube (which need not be dry, but which should not have any drainable water in it). Now add 3 ml. of the dinitrosalicylic reagent, mix and heat for 5 minutes in boiling water. Remove the tube and cool it in cold water. Add distilled water to exactly the 25 ml. mark, place the thumb over the end and invert four or five times to mix. Prepare a blank, using 3 ml. of the dinitrosalicylic reagent and water to the 25 ml. mark. Mix this. Now, using some of the blank solution, set the photoelectric colorimeter* at the null point, using a green filter. Now make a reading, using the unknown solution. Look up on the graph to find out how many mg. of glucose were present.

The graph is prepared by heating 1 ml. solutions containing 0.2, 0.6, 1.0, 1.4, 1.8, and 2.0 mg. of purest glucose with the dinitrosalicylic reagent in the usual manner. The colorimetric readings obtained are plotted on coordinate paper against the mg. of glucose used. A straight line will be obtained.

Having made the colorimetric comparison, calculate the *percentage*

*This refers to the Klett-Summerson colorimeter. See Exp. 49 for directions for the use of a photoelectric colorimeter.

of *glucose* and hand in a written slip with the result and your name, to the instructor.

The dinitrosalicylic acid reagent is prepared as follows: To 300 ml. of 4.5 per cent sodium hydroxide, add 880 ml. of 1 per cent dinitrosalicylic acid and 255 g. of Rochelle salt. To 10 g. of crystalline phenol, add 22 ml. of 10 per cent sodium hydroxide. Add water to dissolve. Dilute to 100 ml. and mix. To 69 ml. of this solution add 6.9 g. of sodium bisulfite and add to the dinitrosalicylic acid solution. Mix well until all of the Rochelle salt has dissolved. Keep tightly stoppered in well filled bottles. The reagent will last for at least one year. Three ml. of the reagent should contain by titration, with phenolphthalein as indicator, the equivalent of 5 to 6 ml. of tenth normal sodium hydroxide.

51. Somogyi-Shaffer-Hartman Quantitative Determination of Glucose (Shaffer, P. A., Somogyi, M., *J. Biol. Chem.* **100**, 695 [1933]).

Be sure you are familiar with the instructions on pages 5-14 before starting this experiment.

Pipette 5 ml. of glucose solution, containing 0.2 to 2.0 mg. of glucose into a large test tube and add exactly 5 ml. of the Shaffer-Somogyi copper reagent. Mix, stopper with a loosely-fitting glass stopper, and place in a boiling water bath for 15 minutes. Remove the tube and cool it in a bath of cold water for about 3 minutes. The temperature of the solution must be brought down to 30 or 40° C. Now add 1 ml. of 5 *N* sulfuric acid, mix and allow to stand for 2 minutes. Titrate with 0.005 *N* sodium thiosulfate until most of the iodine has reacted. Add a few drops of boiled starch solution and titrate carefully until the blue color has disappeared. Now run a blank, heating 5 ml. of distilled water and 5 ml. of the copper reagent, adding sulfuric acid, etc.

The volume of sodium thiosulfate used in titrating the unknown is subtracted from the volume used in titrating the blank. The difference is equivalent to the quantity of cuprous oxide formed by the reducing action of the sugar. Look up in the table on page 41 to find out the amount of sugar to which this corresponds.

The following *precautions* should be observed to obtain satisfactory results with this method. Avoid agitation of the solutions during heating and cooling. Agitation results in excessive oxidation of the cuprous oxide by the oxygen of the air which is shaken into the solutions.

It is undesirable to cool below 30° C. If the 5 ml. of solution

used for a determination contains 1.0 mg. or more of glucose, cooling too far may cause low results because of incomplete oxidation of the reduced copper by iodine after adding sulfuric acid. The reaction is quite rapid until all but 3 to 5 per cent of the cuprous copper is oxidized, but is completed rather slowly at lower temperatures. If the temperature is kept, or raised to, between 30° and 40° C. until the acid is added, the oxidation is complete within 2 to 3 minutes. The excess iodine may then be titrated.

Large amounts of salts lead to different results because they decrease the amount of air in solution. For example, 10 per cent Na_2SO_4 in the sugar solution was found by Somogyi to give results which were 17 per cent too high.

Acid sugar solutions must be neutralized. Carbonates or bicarbonates must *not* be used since they cause comparatively large changes in pH which affect the results greatly.

In this method the essential steps are as follows:

1. The cupric copper is reduced by glucose to give a precipitate of cuprous oxide.

2. The addition of sulfuric acid causes the iodate to react with the iodide to liberate iodine.

3. The cuprous oxide dissolves in the acid and reacts with free iodine as follows:

$$2\,Cu^{+} + I_2 = 2\,Cu^{++} + 2\,I^{-}.$$

Thus a quantity of iodine is used up which is equivalent to the amount of cuprous oxide formed.

4. The iodine which remains is titrated with sodium thiosulfate.

Preparation of reagents: Dissolve 25 g. of anhydrous sodium carbonate and 25 g. of Rochelle salt in 500 ml. of water. Do not heat to dissolve. Add to this 75 ml. of 10 per cent copper sulfate, using a pipette and keeping the tip of the pipette under the solution of carbonate and tartrate in order to avoid losing carbon dioxide. Now add 20 g. of sodium bicarbonate and 5 g. of potassium iodide. Add. 0.1 *N* potassium iodate in volume according to the quantity of sugar you intend to analyze. Usually 200 ml. will do. If this amount is used the blank will amount to about 20 ml. of 0.005 *N* thiosulfate. Dilute the reagent to one liter and mix.

The sodium thiosulfate solution is prepared daily by diluting 25 ml. of 0.1 *N* sodium thiosulfate to 500 ml. The 0.1 *N* iodate contains 3.567 g. of potassium iodate per liter.

The starch solution is prepared by boiling 1 g. of reagent grade soluble starch with 50 ml. of water.

For subsequent modifications of this method see:

Somogyi, M., *J. Biol. Chem.* **117**, 771 (1937).

Note: The values in this table are valid only when 5 cc. of *glucose* solution is heated with 5 cc. of the *copper reagent.* If a smaller volume of sugar solution is used, enough water should be added to make a volume of 5 cc. before adding the copper reagent.

Milligrams of Glucose Corresponding to the Difference between the Titrations Values for the Blank and the Unknown

Ml. of 0.005 *N* Sodium Thiosulfate	Tenths of ml. of 0.005 *N* Sodium Thiosulfate									
	0	1	2	3	4	5	6	7	8	9
0			0.05	0.06	0.08	0.09	0.10	0.11	0.13	0.14
1	0.15	0.16	0.17	0.19	0.20	0.21	0.22	0.23	0.24	0.25
2	0.27	0.28	0.29	0.30	0.31	0.32	0.33	0.35	0.36	0.37
3	0.38	0.39	0.40	0.41	0.42	0.43	0.45	0.46	0.47	0.48
4	0.49	0.50	0.51	0.53	0.54	0.55	0.56	0.57	0.58	0.59
5	0.60	0.61	0.63	0.64	0.65	0.66	0.67	0.68	0.69	0.70
6	0.72	0.73	0.74	0.75	0.76	0.77	0.78	0.79	0.80	0.82
7	0.83	0.84	0.85	0.86	0.87	0.88	0.89	0.90	0.91	0.93
8	0.94	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.04
9	1.05	1.06	1.07	1.08	1.09	1.10	1.11	1.12	1.13	1.15
10	1.16	1.17	1.18	1.19	1.20	1.21	1.22	1.23	1.24	1.26
11	1.27	1.28	1.29	1.30	1.32	1.33	1.34	1.35	1.36	1.37
12	1.38	1.39	1.40	1.42	1.43	1.44	1.45	1.46	1.47	1.48
13	1.49	1.50	1.52	1.53	1.54	1.55	1.56	1.57	1.58	1.59
14	1.60	1.61	1.63	1.64	1.65	1.66	1.67	1.68	1.69	1.70
15	1.71	1.72	1.74	1.75	1.76	1.77	1.78	1.79	1.80	1.81
16	1.82	1.83	1.85	1.86	1.87	1.88	1.89	1.90	1.91	1.92
17	1.93	1.94	1.95	1.96	1.97	1.98	1.99	2.01	2.02	2.03

52. Fermentation Tests. Place in labelled test tubes about 50 mg. of solid glucose, fructose, galactose, maltose, sucrose, and lactose. Add to each tube 5 ml. of fresh yeast suspension (100 ml. of water to 1 small cake of compressed yeast). Shake until the sugar has dissolved in each tube. This will take 4 or 5 minutes. Now place the tubes in a beaker of water at 37° C. and do not move them. Notice that where fermentation occurs the yeast does not settle out. Notice the bubbles of carbon dioxide in the tubes where fermentation is going on. Filter off some of the liquid later, after shaking with a pinch of bone black, and test for ethyl alcohol, using Lieben's test which is capable of detecting 1 part of ethyl alcohol in 2000 parts of water.

53. The Use of the Polarimeter. The polarimeter is a precise optical apparatus and should be treated with care. It is designed to measure the rotation of a plane of polarized light by dissolved substances which have optical activity.

The essential constituent parts of the apparatus are:

1. A light source. For careful work, this must be monochromatic.
2. A polarizer, *i.e.*, a device for producing polarized light.
3. An analyzer, *i.e.*, a device for measuring the rotation of the polarized light.
4. A scale for reading the amount of rotation.
5. A tube for the solution to be measured.
6. Some device for rotating part of the field slightly so that the whole field is not polarized in one plane.
7. A suitable arrangement of lenses so that a sharp field is produced.

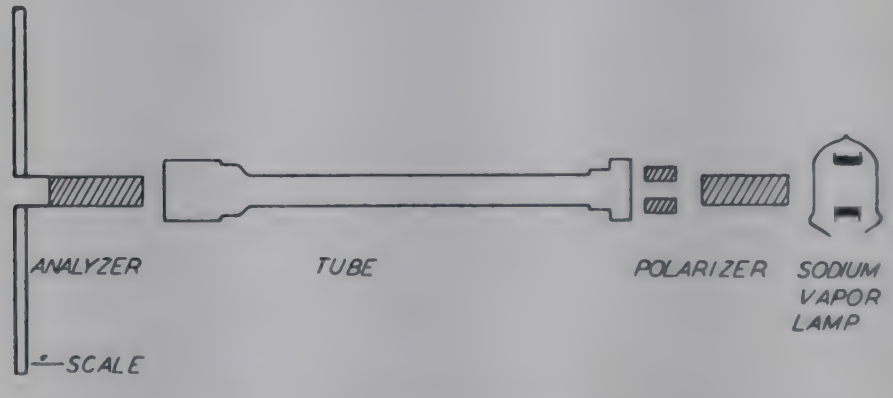


FIG. 5. Diagram of a polarizer.

These parts, except for the lens system, are designated in Fig. 5. The polarizer and analyzer are commonly Nicol prisms. For a description of a Nicol prism refer to texts on physics or optics. The light source is commonly some sort of sodium vapor lamp, or sodium flame, or a mercury vapor lamp provided with suitable filters for isolating one of the bands. The circular scale for measuring the rotation is attached to the analyzer. As this scale is rotated, the analyzing prism is rotated. Neglecting reflection and absorption, the amount of light which passes through the analyzer is determined by the position of this prism with respect to the polarizing prism. This is expressed quantitatively by the following equation, which is a statement of the Malus Law:

$$I = I_0 \cos^2 \theta$$

Here I represents the intensity of the light that passes through the analyzer, I_0 the intensity of the light before striking the analyzer, and "θ" the angle between the planes of polarization of the polarizer and analyzer. When $\theta = 0°$, I will have its maximum value, and when $\theta = 90°$, no light will pass through the analyzer. This is illustrated by the curve in Fig. 6.

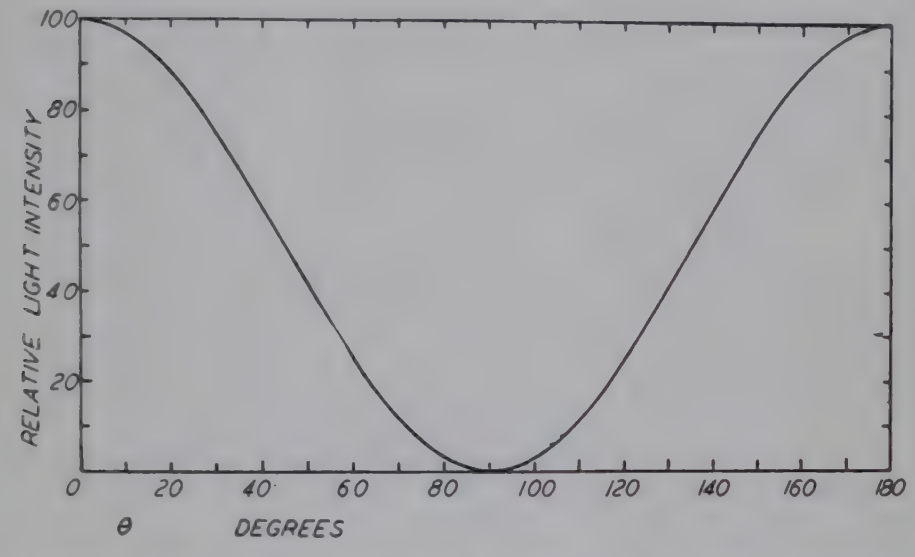


FIG. 6. *Malus Law.* The influence of the angle (θ) between the polarizer and analyzer upon the intensity of the light which emerges from the analyzer.

One could determine the optical rotation of a solution by first setting the analyzer so that no light passed through it when there was no solution in the apparatus. The scale could then be read and the

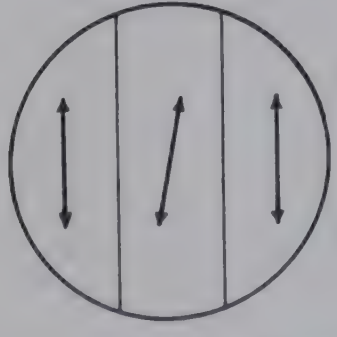


FIG. 7. Planes of polarization in a three-field polarimeter with a 10° angle between the two planes.

solution placed in the apparatus. Again the analyzer could be set so that I had its minimum value. The difference between the two readings would represent the rotation of the solution.

This procedure, as described, would have one very serious handicap. It would be very difficult to determine the minimum value of I, since the curve in Fig. 6 has a rather broad minimum. However, if part of the field is rotated slightly with respect to the rest of the field, this difficulty is overcome. Part of the field may be so rotated by means of the two small Nicol prisms, in addition to the principal polarizing prism, as illustrated in Fig. 5, or by various other means. As a result of such an arrangement, there are different planes of polarization for the separate parts of the field, as illustrated in Fig. 7. The two outside portions of the field have parallel planes of polarization, but the center plane is slightly divergent. As a result, the curve for the intensity, I, is as given in Fig. 8.

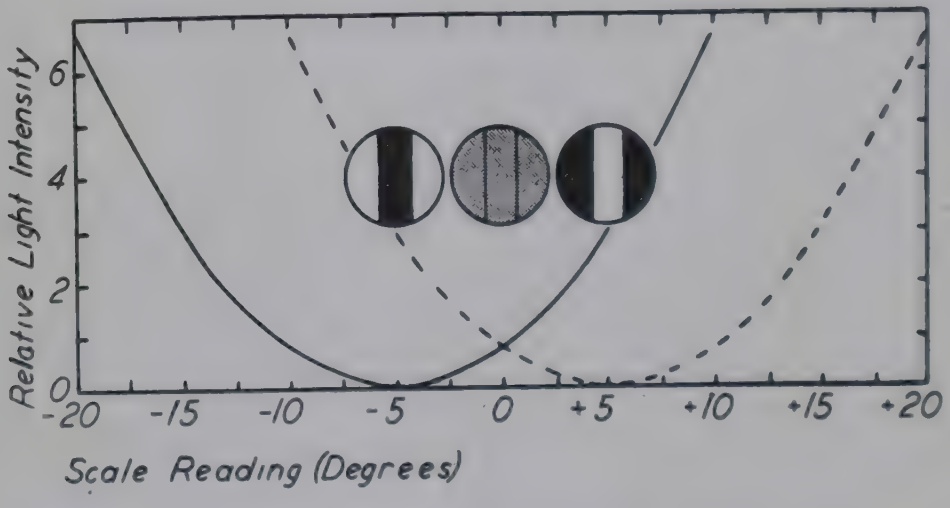


FIG. 8. The result produced by two divergent planes of polarization. The solid line represents the center portion of the field. The broken line represents the outer portions of the field.

Thus there are two curves, one for each plane of polarization produced by the polarizer. The appearance of the field at different settings of the analyzer are indicated by the circles inset in Fig. 8. These two curves intersect at a point. This point can be determined by setting the analyzer so that the field has a uniform intensity. A uniformly dark field should be chosen because of the greater precision with which it can be determined visually.

This explanation should make clear the following directions for determining the rotation of a solution:

1. Turn on the light source and allow it to warm up, if necessary.

A sodium vapor lamp may require twenty minutes, or more, to attain optimum operating temperature.

2. Without a solution in the apparatus, or with the tube filled with water, focus the eyepiece until the field is in sharp focus. This can be done easily if the analyzer is set so that part of the field is dark and part is light.

3. Determine the scale reading when the field is uniformly dark. This is done by rotating the scale with the coarse adjustment until the approximate position is determined. The final adjustment is made using the fine screw below the scale. When the field is uniformly dark, the scale should read zero. Commonly it does not, and the reading must be determined and used in calculating the total rotation of the solution. Make repeated observations until the readings are consistent. Use the average in calculating the results.

4. Place the tube containing the solution in the apparatus. Again focus the eyepiece, if necessary, and determine the scale reading when the field is uniformly dark.

The reading obtained with the solution in place, when corrected for the zero reading, is used to calculate the concentration of the dissolved material. In this calculation, the following relationship is used:

$$[\alpha]_x^{t^\circ} = \frac{100\alpha}{lC}$$

where $[\alpha]_x^{t^\circ}$ is the specific rotation at t° C. with a light of wave length x. If the temperature is 20° C. and a sodium light source is used, the specific rotation is written $[\alpha]_D^{20}$. In the above equation, α is the observed rotation, in degrees; C is the concentration in grams per 100 ml. of solution; and l is the length of solution through which the light passes, in dm.

The wave length of light and the nature of the solvent used influence considerably the value of the specific rotation. The temperature and concentration also influence the rotation, but to a less extent. For the purpose of the present experiment, you may assume a temperature of 20° C. In the following table are given the specific rotations of various carbohydrates in aqueous solution at 20° C. for sodium light. Unless indicated otherwise, these values are for solutions in which $C = 10$. You may use these values for this experiment.

Specific Rotation of Various Carbohydrates

	$[\alpha]_D^{20}$	
L(+)Arabinose	+ 104.5 [1]	
D(−)Fructose	− 92.8 [1]	
D(+)Galactose	+ 80.5 [1]	
D(+)Glucose	+ 52.8 [1]	
Glycogen	+ 194	(C = 0.6) [2]
Inulin	− 33.9	(C = 2) [4]
Lactose	+ 52.5	(C = 5) [1]
Maltose	+ 138.3 [1]	
Sucrose	+ 66.5 [1]	
Starch (amylose fraction)	+ 220	(C = 1) [3]

[1] Compiled from data cited by International Critical Tables, *II*, 334–353, and by Browne, C. A., and Zerban, F. W., Physical and Chemical Methods of Sugar Analysis, 3rd ed., Wiley, 265–272 (1941).

[2] Pringsheim, H., and Will, G., *Ber. deut. chem. Ges.* **61**, 2011 (1928).

[3] Meyer, K. H., Advances in Colloid Science **1**, 152 (1942).

[4] Pringsheim, H., and Reilly, J., *Ber. deut. chem. Ges.* **61**, 2018 (1928).

Determine the rotation of the sugar solution which is provided and calculate the concentration of the solution. Report the result to the instructor.

54. Fructose. Repeat Benedict's, the phenylhydrazine, and Barföd's test with fructose. Why does fructose give the same osazone as glucose? Illustrate with structural formulas.

55. Tashiro and Tietz Test for Fructose. (Tashiro and Tietz, *J. Biol. Chem.* **87**, 307 [1930]). Place 1 ml. of 0.1 per cent bile salt in a test tube and add about 1 ml. of concentrated sulfuric acid and mix. At once add 1 drop of fructose solution and mix. A pink color is produced. Pentoses give an orange color. The pink color is obtained using solutions containing 0.03 per cent of fructose, 0.06 per cent of sucrose, 10 per cent of glucose, 8 per cent of maltose or starch, and 2.5 per cent of dextrin. Other tests for fructose which are somewhat less satisfactory are the Selivanoff test and the Pinoff test.

56. Galactose. Repeat Benedict's and the phenylhydrazine test with galactose. Note that the osazone of galactose is soluble in hot water.

57. The Mucic Acid Test. Place about 500 mg. of solid lactose (galactose also gives the test, but it is too expensive) in a 50 ml. flask. Add 10 ml. of 30 per cent nitric acid (concentrated nitric acid is 69 to 70 per cent), and cover with a watch glass. Heat in the hood on top of the steam bath for 1 hour. Let cool for one hour. Observe crystals of mucic acid. This is a reliable test for galactose and for

carbohydrates containing a galactose group, such as lactose. (Glucose, when present, forms saccharic acid which is too soluble to separate. When considerable amounts of sugar like glucose are present, the mucic acid crystals are very slow to form.) Write structural formulas for mucic acid and saccharic acid showing the different configurations.

Note: Fumes of nitrogen dioxide (red) are *very poisonous.*

58. Arabinose: Bial's Test (modified) (Sumner, J. B., *J. Am. Chem. Soc.* **45**, 2378 [1923]). Place 4 ml. of arabinose solution in a test tube, add about 15 drops of modified Bial's reagent and 4–5 ml. of concentrated hydrochloric acid. Mix, stopper with a wad of cotton, and heat in boiling water for 10 minutes. Observe the blue color. This is a specific test for pentose sugars. Repeat with glucose. Is any blue color produced?

Note: Hexose sugars obscure this test.

The modified Bial's reagent is prepared by dissolving 6 g. of orcinol in 200 ml. of 95 per cent ethyl alcohol. To this is added 40 drops of 10 per cent ferric chloride solution. The reagent keeps for at least six months, whereas the original Bial's reagent will keep for less than a week.

59. Arabinose: Tollen's Test. To 4 ml. of arabinose solution add 3–6 drops of 5 per cent alcoholic phloroglucinol solution. Add 4 ml. of concentrated hydrochloric acid, mix, and bring just to boiling. Observe the momentary production of a pink color. *Quickly* observe the absorption band with the spectroscope. This test is specific for *pentose* sugars.

Use of a Pocket Spectroscope. The pocket spectroscope is a convenient direct vision spectroscope. It can be used for observing emission or absorption spectra. To use such a spectroscope, adjust the slit until it is nearly closed. Then adjust the draw tube until the main Fraunhofer lines can be seen distinctly when the instrument is directed toward a bright sky. For use with deeply colored solutions the slit may have to be opened wider.

60. Feulgen Test for 2-Ribodesose. Heat 1 ml. of the solution suspected of containing 2-*d*-ribodesose in combined form, with 6 to 8 ml. of 0.05 *N* hydrochloric acid for 1 to 6 minutes in boiling water. Cool and add 2 drops of dinitrophenol and 0.1 *N* sodium hydroxide until the first yellow color appears. Add an equal volume of citrate buffer and mix. To 2 to 3 ml. of this solution add 0.5 to 1.0 ml. of Schiff reagent and mix. A red color will appear if as much as 0.5 mg. of thymonucleic acid (containing 2-*d*-ribodesose) was originally present.

The color develops very slowly and should be observed for several hours.

The citrate buffer contains 230.5 ml. of 0.1 *M* trisodium citrate and 69.5 ml. of 0.1 *N* hydrochloric acid.

The Schiff reagent is made by mixing 1 g. of fuchsin with 30 to 40 ml. of 1 *N* hydrochloric acid and 2 to 3 g. of sodium bisulfite. The reagent should have only a faint pink color.

61. Arabinose. Try Benedict's and the dinitrosalicylic tests with arabinose. Prepare the osazone of arabinose and observe the crystals under the microscope.

62. Maltose. Repeat Barföd's, Benedict's, and the phenylhydrazine tests with maltose.

63. Lactose. Repeat Barföd's, Benedict's and the phenylhydrazine tests with lactose. What is the taste of solid lactose? Is lactose soluble in 95 per cent ethyl alcohol?

Sucrose. Sucrose, saccharose, or cane sugar crystallizes readily as monoclinic crystals. Repeat Barföd's, Benedict's, and the dinitrosalicylic acid tests with a fresh solution of sucrose. Will sucrose form an osazone? Observe crystals of sucrose under the microscope using a low-power objective. Try the dinitrosalicylic test after boiling 1 ml. of sucrose solution for a few seconds with one drop of glacial acetic acid and then neutralizing with 2 drops of concentrated sodium hydroxide. Is sucrose easily hydrolyzed? What products are formed?

65. Invertase (Sucrase, Saccharase). Add a pinch of acetone yeast powder, or else a drop of commercial *invertase* solution, to 1 ml. of sucrose solution and allow to stand for 1 minute. Now apply a reduction test. Has the sucrose been hydrolyzed by the enzyme?

Invertase can be made by triturating compressed yeast with a large excess of acetone in order to rupture the cell walls. The product is washed with ether and then dried in a desiccator. If allowed to dry in the air, it becomes hard and horny and difficult to suspend in water.

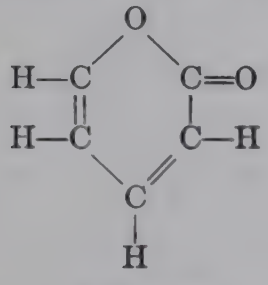
66. Prunasin. The bark of the wild cherry tree contains the glucoside prunasin, a compound of benzaldehyde, hydrocyanic acid and *β-d*-glucose, in other words, a cyanogenetic *β*-glucoside. Place a few cut-up pieces of cherry bark in water and add a pinch of defatted almond meal. Mix and allow to digest for 10 to 20 minutes. Now test for hydrocyanic acid. The reagent used is alcoholic benzidine plus very dilute copper sulfate plus excess of water. This gives a plum colored precipitate with hydrocyanic acid.

Prunasin is hydrolyzed by the *β*-glucosidase in the almond meal.

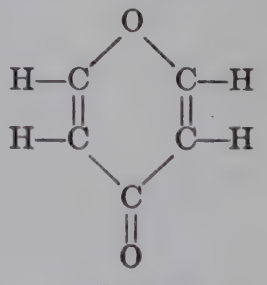
Whether or not a second enzyme, nitrilase, is present is debatable.

67. Starch. Boil a *small* pinch of starch after it has been shaken up well with 10 ml. of water. Cool and add a drop of iodine solution. Heat and notice that the blue color disappears and reappears upon cooling if all of the iodine has not been driven off. (*Note:* If too much iodine is added, the blue color will not disappear on heating.) Does sodium hydroxide interfere with this test?

The reaction of iodine with starch is a physical reaction and not a chemical one. It depends upon adsorption of the iodine by the starch. Barger (Barger, G., Some applications of Organic Chemistry to Biology and Medicine, p. 161 [1930]) states that compounds that contain the α- or γ-pyrone ring will give a blue color with iodine provided the substance forms a colloidal solution in water.



α-Pyrone



γ-Pyrone

The coumarins, saponarin, acid-treated cellulose, cholalic acid, narcein, and euxanthic acid give color with iodine.

68. Digestion of Starch by Salivary Amylase. Cool about 10 ml. of well-boiled starch solution (*Note:* Do not try to boil thick starch over a free flame. The thick colloidal solution will cause the beaker to break), to about 40° C. and add about 1 ml. of your saliva. Mix. Remove a 1 ml. portion every 2 or 3 minutes and test with a drop of iodine solution. Observe the formation of erythro-dextrin, which gives a red color with iodine. When no color is given with iodine, show by a reduction test that maltose is present.

69. Precipitation of Starch by Tannic Acid.* Add some fresh tannic acid solution to some boiled starch.

70. Indiffusibility of Starch. Place some boiled starch solution within a cellophane membrane, and suspend in a beaker of water. After some time, add iodine solution to both the outer and inner liquids. Will starch diffuse through the membrane?

*The tannic acid usually employed in laboratories is also called tannin and gallotannic acid. It is obtained from *Turkish* or *Chinese* nutgalls. It is possibly penta-digalloyl-glucose, $C_{76}H_{52}O_{46}$, mol. wt. 1700. Many other tannins exist.

71. Inulin. Does inulin give any color with iodine? Compare with a blank containing exactly the same number of drops of iodine solution.

Does inulin (like starch) exist as granules?

Will saliva digest inulin? Test afterwards for a reducing sugar.

72. Inulin: Hydrolysis by Acid. Boil 2 ml. of inulin solution with 2 drops of concentrated hydrochloric acid. Neutralize with 4 drops of concentrated sodium hydroxide and test for a reducing sugar. Inulin is the anhydride of what monosaccharide?

73. Dextrin. Does dextrin reduce? What color does it give with iodine? Is dextrin precipitated by adding alcohol to 50 per cent concentration? To 80 per cent concentration?

The following equation can be used for diluting the alcohol:

$$(X + Y)Z = 95Y$$

where,

X = volume of water taken, and

Y = volume of 95 per cent alcohol that will have to be added to give a concentration of Z volumes per cent.

This neglects the contraction that occurs when alcohol is mixed with water. Water can be added to make the final volume equal to the amount calculated.

A simple way of using this equation can be illustrated by a specific example. To prepare 50 per cent alcohol from 95 per cent, dilute 50 ml. of 95 per cent alcohol to 95 ml. with water.

74. Precipitation of Dextrin and Starch. Make about 10 ml. of a solution of dextrin and boiled starch. Saturate this solution with solid ammonium sulfate. Is the starch precipitated?

Note: When ammonium sulfate is dissolved in water it causes a considerable fall in temperature. It will therefore be necessary to warm the solution until it reaches room temperature.

Does tannic acid precipitate dextrin?

75. Hydrolysis of Cellulose. Submerge a wad of cotton in about 2 ml. of concentrated sulfuric acid. Stir until dissolved. Dilute by pouring into 30 volumes of cold water and boil for a few minutes. Neutralize with concentrated sodium hydroxide and test for glucose. Cellulose is an anhydride of glucose. The disaccharide cellobiose is one of the intermediate products of hydrolysis.

76 Cellulose Acetate. Place a small wad of cotton in a dry test tube and add 7 ml. of glacial acetic acid, 2 ml. of acetic anhydride, and 1 drop of concentrated sulfuric acid. Mix well and allow to stand for

3 or 4 hours, or longer. When the cellulose has all dissolved, precipitate the cellulose acetate by pouring the solution into an excess of water. When dried the material will dissolve in chloroform.

77. Nowopokrowsky Test. Place on a piece of filter paper a drop of Nowopokrowsky's reagent (iodine, potassium iodide, zinc chloride solution). Is a blue color produced? Will cellulose (filter paper) give any blue color if the zinc chloride is not present in the reagent?

To prepare Nowopokrowsky reagent (Rawlins, T. E. Phytopathological Methods, John Wiley and Sons, p. 39 [1932]) dissolve 50 g. of zinc chloride and 16 g. of potassium iodide in 17 ml. of water. Add an excess of iodine and allow to stand for several days: Use the supernatant liquid.

78. Lignin Test. Dip a match into a solution of phloroglucinol in concentrated hydrochloric acid. Note the pink color. This is a test for lignin. Will pure cellulose give this test (use filter paper)?

79. Cuprammonium Solution. Dissolve some cellulose in about 5 ml. of cuprammonium solution and re-precipitate by adding 10 per cent sulfuric acid in excess.

Note: The cuprammonium solution, Schweizer's reagent, can be prepared (Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th ed., Blakiston p. 1233 [1947]) by adding to ten parts of ammonium hydroxide, sp. gr. 0.90, three parts of distilled water and a slight excess of cupric carbonate. Shake well and centrifuge. Decant and use the clear solution. This is the best method of preparing the reagent. But it can also be made by precipitating cupric hydroxide by adding ammonia to a solution of cupric sulfate. The precipitated cupric hydroxide is filtered off, washed with water and dissolved in concentrated ammonium hydroxide.

Other reagents that dissolve cellulose are metallic chlorides dissolved in hydrochloric acid. Cross and Bevan's reagent for dissolving cellulose is made by dissolving one part of zinc chloride in 2 parts (by weight) of concentrated hydrochloric acid.

Rayon (which is widely used at the present time as a substitute for silk) is made by shaking wood pulp (cellulose) with sodium hydroxide and carbon disulfide. The cellulose forms a colloidal sol known as cellulose xanthate. This solution is filtered and forced through very fine glass tubes into a solution of acid. The cellulose is precipitated in fine threads which are reeled in under tension.

80. Gum Arabic. Is gum arabic hydrolyzed by boiling with 5 per cent hydrochloric acid? What is the product?

81. Agar-agar. Of what monosaccharide is agar-agar composed? Try the *mucic acid* test.

82. Carbohydrate Unknowns. Apply for 2 carbohydrate unknowns by handing in 2 test tubes, labelled with your name and numbered 1 and 2, respectively. The unknowns may contain no more than one polysaccharide, one disaccharide and one monosaccharide.

The first thing to do in this analysis is to examine a small sample of the dry specimen under the microscope. Sucrose, lactose, and starch (granules) are easy to detect. Starch granules may be easily recognized if a small portion of the unknown is mounted in diluted Lugol's solution and then examined under the microscope.

To detect arabinose (or xylose) in the presence of other sugars it is best to extract the solid unknown by shaking about 0.5 g. with 5 ml. of 95 per cent alcohol. Use a dry flask and a dry funnel. Filter and add 5–10 drops of the filtrate to 5 ml. of water and use this for Bial's test for arabinose. *Note:* Galactose will interfere with this test by giving a green color if the Bial's test is heated for over 10 minutes, or if any water is present in the 95 per cent alcoholic extract.

Dextrin (if present together with a starch) can be extracted with cold water. The solution is filtered and dextrin tested for by adding iodine solution to the filtrate. In case of doubt try a blank of distilled water, adding exactly the same number of drops of iodine solution to the unknown and to the blank.

To detect sucrose dissolve about 200 mg. of solid unknown in about 100 ml. of water and place 1 ml. portions in two Folin-Wu sugar tubes. To tube No. 1 add a very small drop of invertase solution and mix. After a few minutes add to both tubes 3 ml. of the dinitrosalicylic reagent. Add to tube No. 2 a very small drop of invertase solution only after having first added the dinitrosalicylic reagent. Heat both tubes in boiling water for 5 minutes. If sucrose was present it will have been hydrolyzed by the invertase, and tube No. 1 will be darker than tube No. 2. The invertase added to tube No. 2 will not affect the sucrose because it will be inactivated by the dinitrosalicylic reagent, which is alkaline. Invertase is added to both tubes to eliminate any difference due to reduction by it.

To detect disaccharides such as maltose, sucrose, or lactose place 1 ml. of the solution containing about 200 mg. of solid unknown in 100 ml. of water in a test tube. Add 3 ml. of Barföd's reagent, mix, and heat in boiling water for 10 minutes. A reduction indicates

monosaccharides, or sucrose, but with a moderate quantity of monosaccharide present there may be little or no reduction visible. Now filter and add to the clear filtrate 5 ml. of Benedict's reagent. Heat in boiling water 5 minutes. A reduction indicates maltose, sucrose, or lactose.

The table below indicates which of the tests, lettered *A*, *B*, *C*, etc., are given by various common sugars.

Carbohydrate Tests with Unknowns

A = Benedict's or D. N. S. test before fermentation.
B = Benedict's or D. N. S. test after fermentation.
C = Bial's test.
D = Invertase test.
E = Barföd's test.
F = Benedict test applied after Barföd and filtering.
G = Alcoholic precipitation of lactose.
H = Tashiro and Tietz test.

1 Arabinose	*2* Galactose	*3* Fructose	*4* Glucose	*5* Lactose	*6* Sucrose	*7* Maltose
A+	A+	A+	A+	A+	A−	A+
B+	B+	B−	B−	B+	B−	B+
C+	C−	C−	C−	C−	C−	C−
D−	D−	D−	D−	D−	D+	D−
E+	E+	E+	E+	E−	E+	E−
F−	F−	F−	F−	F+	F+	F+
G−	G−	G−	G−	G+	G−	G−
H−	H−	H+	H−	H−	H+	H−

8 Sucrose + Glucose	*9* Sucrose + Fructose	*10* Sucrose + Galactose	*11* Sucrose + Arabinose	*12* Maltose + Glucose	*13* Maltose + Fructose
A+	A+	A+	A+	A+	A+
B−	B−	B+	B+	B+	B+
C−	C−	C−	C+	C−	C−
D+	D+	D+	D+	D−	D−
E+	E+	E+	E+	E+	E+
F+	F+	F+	F+	F+	F+
G−	G−	G−	G−	G−	G−
H+	H+	H+	H+	H−	H+

14 Maltose + Galactose	*15* Maltose + Arabinose	*16* Lactose + Glucose	*17* Lactose + Fructose	*18* Lactose + Galactose	*19* Lactose + Arabinose
A+	A+	A+	A+	A+	A+
B+	B+	B+	B+	B+	B+
C−	C+	C−	C−	C−	C+
D−	D−	D−	D−	D−	D−
E+	E+	E+	E+	E+	E+
F+	F+	F+	F+	F+	F+
G−	G−	G+	G+	G+	G+
H−	H−	H−	H+	H−	H−

To detect lactose dissolve about 500 mg. of solid unknown in

2 ml. of water. Add 20 ml. of 95 per cent alcohol and at once pour upon a dry filter. Catch the filtrate in a dry test tube. After standing for 20 minutes the lactose in the filtrate will crystallize out and *will stick to the glass walls of the test tube.*

Still further evidence regarding the nature of an unknown can be obtained by mixing 50 ml. of solution (containing 100 mg.) with a half cake of compressed yeast and, after rotating to secure an even suspension of yeast, allowing the material to stand at room temperature for 4–8 hours. The solution may be left in the ice chest until the next laboratory period, if necessary. The solution is then filtered, after shaking with a quarter teaspoonful of Lloyd's reagent (Celite may be used instead of Lloyd's reagent), and 1 ml. of filtrate is tested for reduction by the dinitrosalicylic acid method. If lactose, galactose, maltose or arabinose were present, there would be a reduction.

Note: The combination of sucrose with glucose gives the same tests as the combination of sucrose with fructose. Hence the combination of sucrose with fructose will not be given as an unknown. Likewise the combination of lactose with glucose gives the same tests as the combination of lactose and galactose. The combination of lactose and galactose will not be given as an unknown. Ordinary yeast cakes do not ferment maltose. Hence, maltose and glucose together give the same tests as the combination of maltose and galactose. The latter combination will not be given.

83. Starch, Quantitative Determination of. Weigh out about 1 g. of a sample of special dry potato starch furnished by the instructor. Weigh the sample as accurately as possible from a weighing bottle and place it in a dry Kjeldahl flask. Add 200 ml. of 5 per cent sulfuric acid. The dry starch should be placed in the flask and the traces adhering to neck of the flask should be rinsed into the flask with the acid. Clamp the flask to a ring stand and support in its neck, with another clamp, a 25 × 200 mm. test tube condenser supplied with running water (see Fig. 9). Boil gently for 2 hours. It is advisable to heat the starch solution rather slowly, with shaking, until it begins to boil. After 2 hours, exactly neutralize the solution with normal sodium hydroxide. Avoid an excess of alkali. Dilute to 1 liter and mix.

Determine the amount of glucose present in the neutralized hydrolyzate using either the Sumner dinitrosalicylic method, or the Somogyi-Shaffer-Hartman method. The weight of glucose multiplied by 0.9 should equal the weight of the starch. Does the result agree with your weighing?

Glycogen can be determined by the same method used to determine starch. Here, however, the factor used is 0.926. The glycogen should be boiled for 3 hours with 2.2 per cent hydrochloric acid.

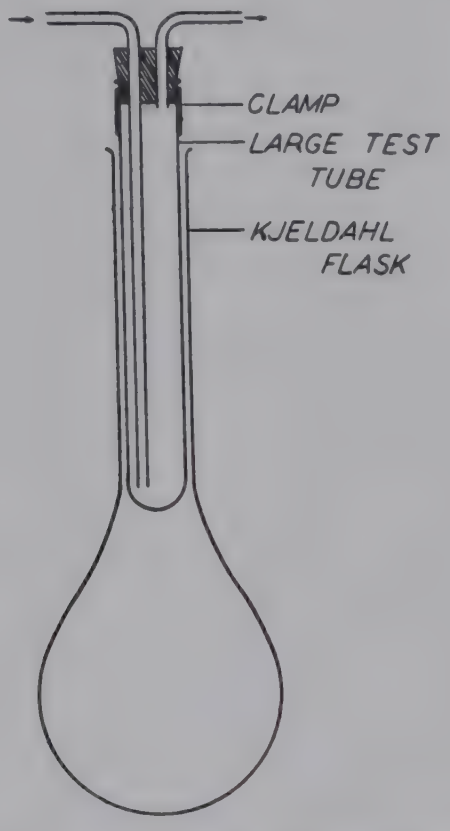


FIG. 9. Apparatus for starch hydrolysis.

84. Glycogen, Preparation from Liver (Sahyun, M., and Alsburg, C. L., *J. Biol. Chem.* **89**, 33 [1930]). Grind 24 g. of *fresh* rabbit or beef liver in the meat grinder. Rub the ground material in a mortar with 75 ml. of 3 per cent trichloroacetic acid. Strain through cheesecloth. Repeat the extraction with trichloroacetic acid. Centrifuge the extract. To the supernatant add two volumes of 95 per cent ethyl alcohol. Decant a part of the clear supernatant and centrifuge down the glycogen in the rest. Dissolve the glycogen in a little 3 per cent trichloroacetic acid and centrifuge down the protein. Again precipitate the glycogen with alcohol and centrifuge. Repeat the dissolving in trichloroacetic acid and the precipitating with alcohol twice more. Now dissolve the glycogen in the least amount of water and filter with suction. Precipitate the glycogen again with alcohol. Wash it with 95 per cent alcohol and then with absolute alcohol. Dry in a vacuum over calcium chloride.

The yield should be about 1.8 g. from 24 g. of fresh rabbit liver.

It will be 2 weeks before the glycogen is dry. The ash content of the glycogen will be from 0.18 to 0.20 per cent, and 16 per cent of this ash will be P_2O_5. Glycogen is easily altered by heating to 105° C. Dissolve a little glycogen in water and add an equal volume of saturated ammonium sulfate. Now add a drop of dilute potassium tri-iodide solution (Lugol's solution). Observe the reddish brown color.

85. Pectin Preparation. Put one-half a fresh grapefruit peel (100 to 120 g.) through a meat chopper. Stir up with water and wash several times on a wad of cotton in a large filter funnel. Now transfer to a 1000 ml. flask, using 500 ml. of water. Boil very gently for 2 hours over a very low flame, avoiding too great a concentration. Cool and filter through a wad of cotton into another flask or beaker and evaporate the extract to about 100 ml. volume.

Add 300 ml. of alcohol to precipitate the pectin. Filter off the pectin and allow it to dry on the filter paper.

The formation of a pectin solution from grapefruit peel will require only 30 minutes if one employs 0.05 *N* HCl instead of water.

Pectin is present in cell walls as the insoluble "protopectin." Boiling converts this to the soluble "pectin." Pectin is a polygalacturonic acid that is partly methylated. Crude pectin contains impurities containing galactose and arabinose.

Many enzymes are known which attack pectin. Pectinase dissolves pectin by splitting the chain. Pectase causes the removal of methyl alcohol, leaving pectic acid and forming a gel under the proper conditions.

86. Pectin Gel by Pectase Action. Pour 3 to 4 ml. of commercial pectin solution (Certo) into a test tube and neutralize by adding 0.1 *N* NaOH with shaking until the natural indicator present gives a black color. The indicator is yellow when acid, black when neutral, and red when alkaline. Frequently commercial preparations do not contain enough of this indicator. In this case a little Phenol red indicator solution can be added. Add a pinch of jack bean meal or 2 or 3 ml. of potato juice to supply the pectase. Mix and allow to stand. The reaction will turn acid, and the pectin will form a gel. After standing 24 hours the gel will shrink with the separation of serum. This is "*syneresis*" and is shown also with fibrin and with paracasein.

87. Pectin Gel with Cane Sugar. To 3 ml. of undiluted, unneutralized Certo add as much cane sugar as you can dissolve. Allow

to stand. Does a jelly form? Is it soluble in water? Does it show syneresis?

Note: Exps. 86 and 87 can be carried out with the pectin made by you. The pectin solution must be neutral for pectase to form a gel, and acid for cane sugar to form a gel. The acid added should be citric or tartaric. The action of pectase will not produce a gel if calcium ions are absent.

PLANT-PIGMENTS

88. Anthocyanins. Extract 1 g. of either dried hollyhock petals, from deep red flowers, or red rose petals with 40 ml. of 95 per cent ethyl alcohol, to which has been added 4 drops of concentrated HCl. After standing for 6 to 12 hours, filter, and press out as much of the liquid as possible from the petals. Add 3 ml. of distilled water to the alcoholic extract and then add 120 ml. of benzene to precipitate the anthocyanin in a concentrated aqueous solution. Let the material stand until the aqueous layer has separated. This may require an hour or more.

Decant most of the benzene layer (but be very careful not to spill any benzene about the laboratory!) and then add enough water to make the aqueous layer up to a volume of about 10 ml. Transfer to a separatory funnel. Draw off the aqueous layer and discard the remaining benzene layer. Now extract the aqueous layer with a 30 ml. portion of ether. Discard the ether layer, which will be pink. Add enough water to the aqueous layer to make a volume of 10 ml. once more, and again extract with ether, using 20 ml. After this second ether extract has been removed, aqueous ammonia (dilute) may be added to it to show the presence of flavones or "water soluble yellows." A yellow color in the ammonia layer indicates flavones, and a yellow color in the ether indicates "water soluble yellows." Finally extract the aqueous anthocyanin layer a third time with ether, using 20 ml.

Again make up the volume of the anthocyanin layer to 10 ml. and extract with 15 ml. of isoamyl alcohol. Repeat. This removes free anthocyanidins, and also a small portion of the anthocyanin, since diglucosidic anthocyanins are slightly soluble in the amyl alcohol.

Now remove the aqueous anthocyanin layer and add a solution of basic lead acetate until the color just has changed to blue. Precipitate the lead salt of the anthocyanins by the addition of two

volumes of 95 per cent ethyl alcohol. Let stand for 15 minutes and then centrifuge down the precipitated lead salt. Remove the supernatant liquid and add to the precipitate 5 ml. of 5 per cent HCl. Filter off the $PbCl_2$, using as small a piece of filter paper as possible. Wash once with a little water. Do not allow the volume of the solution to increase over 7 or 8 ml. Now heat over the steam bath or in a beaker of boiling water for one hour. The solution must not be allowed to evaporate to a volume of less than 5 ml. or sugar will be destroyed.

Cool the solution, dilute to 10 ml., and extract with 20 cc. of isoamyl alcohol. Repeat the extraction without again diluting the aqueous solution. This should remove most of the color from the aqueous layer, since the anthocyanins have been hydrolyzed by heating with acid and the anthocyanidins go into the amyl alcohol, leaving most of the sugar in the aqueous layer.

Separate the aqueous layer and add solid sodium acetate until the solution is no longer acid to Congo red paper. Now add 1 ml. of phenylhydrazine acetate solution. Heat for half an hour in boiling water, and then cool. An osazone should separate, probably as more or less amorphous material. The osazone crystals may be centrifuged down, dissolved in a few drops of alcohol, and again centrifuged. The alcohol is decanted and water is added dropwise until a slight clouding occurs. The material is then boiled a minute or two very gently and is allowed to cool. Good crystals of the osazone will separate.

Petals of the hollyhock, *Althea rosea*, probably contain mono- and diglucosides of the anthocyanidins; malvidin, petunidin, and delphinidin. In general, hexoses, pentoses, or disaccharides can occur in combination with the anthocyanidins. Flavones also occur as glucosides or in combination with other sugars. The anthocyanins are indicators. Petals of the red rose (*Rosa gallica*) contain glucosides of cyanidin.

Free flavones may be extracted from yellow or white flowers with ether, alcohol, or dioxane, to give dilute solutions. Addition of ammonia produces a yellow color. If ether has been used, the flavones will go into the aqueous ammonia layer. If the yellow color remains largely in the ether, the "water soluble yellows" of unknown composition are causing the color.

References: Gortner, R. A., Outlines of Biochemistry, 2nd ed., Wiley and Sons, pp. 753–766 (1938); Link, K. P., in Gilman, H., Organic Chemistry, Wiley and Sons, pp. 1114–1137 (1938); Shriner,

R. L., and Moffett, R. B., *J. Am. Chem. Soc.* **61**, 1474 (1939); **62**, 2711 (1940).

89. Chlorophyll. Grind 25 g. of fresh spinach leaves in a mortar with a small amount of sand until a pulp has been formed. The leaves should be freed of petioles and large veins. Extract the pulp for one hour with 50 ml. of dioxane and then squeeze out the extract through a piece of cheesecloth. Re-extract the pulp for an hour with 50 ml. more of dioxane. Again squeeze out the extract through cheesecloth. Combine the two extracts and filter. Now add 30 ml. of water to the filtrate to precipitate the chlorophylls. The last amount of water should be added in small portions and only just enough should be added to flocculate the chlorophylls.* An excess of water should be avoided, but sometimes 30 ml. is not enough.

Filter off the precipitated chlorophylls. The filtrate contains carotenoids, flavones and other water-soluble yellow pigments. The carotenoids in the filtrate are mostly xanthophyll (lutein). Carotene is very largely precipitated with the chlorophylls. Discard this filtrate.

Dissolve the precipitate of chlorophylls in 25 ml. of dioxane. The filter paper containing the chlorophylls may be added directly to the dioxane, and the ground up filter paper can be filtered off after the chlorophylls have been dissolved. Add enough water to the filtrate to precipitate the chlorophylls.* About 15 ml. should be required. Filter off the precipitated chlorophylls.

Cut the filter paper containing the chlorophylls in half. Dissolve the chlorophyll on one-half in 10 ml. of 92 per cent methyl alcohol. Add an equal volume of petroleum ether and shake. Chlorophyll A will be in the upper layer and chlorophyll B in the lower layer. Separate the two layers. Wash the chlorophyll A solution with another 10 ml. portion of 92 per cent methyl alcohol. Combine the washing with the chlorophyll B solution. Compare the two solutions of chlorophyll. Observe the fluorescence. Observe the absorption spectra.

To one-half of the petroleum ether solution of chlorophyll A, add 2 ml. of a 30 per cent solution of KOH in methyl alcohol. Immediately observe the color changes in the alcoholic layer, particularly near the interface between the two solutions. In which layer is the final green product? What is the product? The intermediate color change is known as the phase test. Explain the changes which occur.

* The addition of a small amount of solid NaCl will help to flocculate the chlorophylls.

Can all of the green color be extracted from the petroleum ether layer by adding more methyl alcohol or water?

To the methyl alcohol solution of chlorophyll B add 10 ml. of ethyl ether. Wash the resulting solution with several 10 ml. portions of water to remove the methyl alcohol. Rotate the solution gently to wash with the water. Do not shake it vigorously, or an emulsion may be formed. More ether should be added as required to maintain a volume of 10 ml. in the ether layer. Compare the washed ether solution with the petroleum ether solution of chlorophyll A. To one-half of the ethyl ether solution add 2 ml. of 30 per cent KOH in methyl alcohol and observe the phase test. Compare with the phase test given with chlorophyll A.

Dissolve the chlorophylls on the other half of the filter paper in 10 ml. of 30 per cent KOH in methyl alcohol. Filter if necessary and heat on the steam bath, or in hot water in a beaker, for 45 minutes. The heating should keep the methyl alcohol boiling, but excessive evaporation should be avoided. It is advisable to add piece of coarse carborundum to prevent bumping. Occasionally add more methyl alcohol, if necessary. After 45 minutes, cool the methyl alcohol solution and add 10 ml. of water. Observe the color. Is the solution fluorescent? Just acidify the solution with hydrochloric acid. It should be made alkaline to Congo red paper, but acid to litmus paper. Extract with two 10 ml. portions of ethyl ether. If the solution is neutral, or just faintly acid, the magnesium-free pigments will go into the ether layer.

Extract the ether solution with two 10 ml. portions of 4 per cent hydrochloric acid. This extracts the phytochlorin e. From which chlorophyll is this formed? If the remaining ether layer is less than 10 ml. in volume, dilute it to 10 ml. with ether and extract with two 10 ml. portions of 15 per cent hydrochloric acid. This extracts the phytorhodin g. From which chlorophyll is this formed?

What are the pigments in the alcoholic KOH solution before acidifying? Outline the reactions leading to the formation of phytochlorin e and phytorhodin g.

90. Carotenoids. Grind 2 g. of dried spinach leaves in a mortar. Add 50 ml. of petroleum ether. Allow to stand for at least 15 minutes with occasional stirring. Cover the mortar with a watch glass to avoid excessive evaporation. Filter the resulting solution. Add 2 ml. of a 30 per cent solution of KOH in methyl alcohol to the filtrate. Mix, and allow to stand for at least an hour, or better still, until the

next laboratory period. (Store the mixture in the cold room between laboratory periods.) Add 50 ml. of water and separate in a separatory funnel after shaking briefly. Extract the petroleum ether solution with several more portions of water. Do not shake the petroleum ether and water together too vigorously, or an emulsion will be formed.

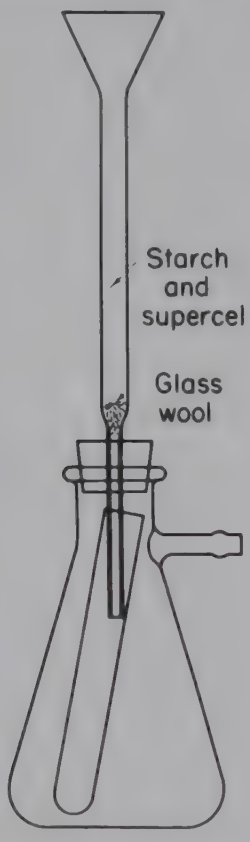


Fig. 10. Tswett column.

What are the pigments in the aqueous extract? Separate the carotene and xanthophyll in the petroleum ether solution by one of the two following methods: (1) Extract the resulting petroleum ether solution with 50 ml. of 92 per cent methyl alcohol, using two 25 ml. portions. Xanthophyll will be extracted by the alcohol, and carotene will be left in the petroleum ether. (2) The carotene and xanthophyll can also be separated by means of a Tswett column (see Fig. 10). Place a small amount of glass wool in the bottom of the column and fill the column about three-fourths full with a mixture of soluble starch and Supercel (2 parts of starch, Eimer and Amend, indicator grade, to 1 part of Hyflo Supercel, by weight). Dry the petroleum ether extract with anhydrous sodium sulfate and pull it through the column with suction. Add more petroleum ether to "develop" the column until the carotene comes through into the test tube.

Evaporate the solution of carotene to dryness on the steam bath. Avoid excessive heating of the dry residue. Dissolve the residue in 5 ml. of *dry* chloroform. Observe the absorption spectrum and perform the Carr-Price test. The Carr-Price test is performed by adding about one-half ml. of the solution to about 2 ml. of the Carr-Price reagent in a *dry* test tube. Observe the color.

Transfer the xanthophyll to petroleum ether in the same way that chlorophyll B was transferred from methyl alcohol to ethyl ether. Use 10 ml. of petroleum ether. Observe the absorption spectrum and color. Compare with carotene.

The Carr-Price reagent is prepared by dissolving 20 g. of dry antimony chloride in 100 ml. of chloroform dried with P_2O_5. This reagent has been widely used in the past for the determination of vitamin A and provitamins A.

91. Preparation of Crystalline Bixin. Extract 10 g. of annatto powder (Fisher Scientific Co.) with 100 ml. of hot acetone (danger) for 10 minutes and decant the liquid through a filter. Re-extract the residue with another 100 ml. portion of hot acetone for the same length of time and decant the liquid through the same filter. Evaporate the combined filtrates, in the hood, to about 50 ml. (on the steam bath). Place the solution in the cold room until the crystallization of bixin has taken place. Filter off the crystals and wash them several times with 95% ethyl alcohol, until the washings are almost colorless. Dry the crystals. The dried crystals should have a dark, reddish purple, metallic luster; m.p. 192–4° C.

Dissolve some of the bixin crystals in carbon disulfide (danger) to give a sufficiently dilute solution and determine with a spectroscope or spectrophotometer the number and location of the absorption bands.

VITAMIN C

92. Vitamin C-Quantitative Determination of:

Using the sample of orange juice, lemon juice, or urine given by the instructor, determine the amount of ascorbic acid (vitamin C) present.

Use a titration method. The student is required to look up the details of a method and to make up and standardize his own reagents. The following references are suggested:

Bessey, O. A., and King, C. G., *J. Biol. Chem.* **103**, 687 (1933).

Musulin, R. R., and King, C. G., *J. Biol. Chem.* **116**, 409 (1936).

GASTRIC ANALYSIS

93. Free Hydrochloric Acid. Test the sample of gastric contents for free hydrochloric acid, using Congo red paper and dimethylamino azobenzene paper.

Pour about 2 ml. of *fresh* Günsburg's reagent into a clean evaporating dish and dry on a steam bath. Wet a glass rod with the sample of gastric contents and draw a line through the dried Günsburg reagent with the wet rod. Now heat the material on the steam bath once again. If a red streak is formed, free hydrochloric acid was present in the gastric contents.

Test combined hydrochloric acid (Witte's peptone and a little tenth normal hydrochloric acid) with the above reagents. Is hydrochloric acid neutralized by peptone?

Test 2 per cent acetic acid with the above reagents. Acetic acid furnishes only small quantities of hydrogen ions.

Günsburg's reagent is made by dissolving 2 g. of phloroglucinol and 1 g. of vanillin in 100 ml. of 95 per cent alcohol. It must be made up fresh every two months.

Note: Artificial gastric contents can be made by adding to 2 liters of water, 20 ml. of concentrated hydrochloric acid, 20 ml. of 85 per cent lactic acid, 5 g. of 1 : 3000 U.S.P. pepsin, 10 ml. of blood, and some cracker crumbs.

94. Pepsin. Is pepsin present in the sample of gastric contents? See page 116 for directions for the detection of pepsin.

95. Lactic Acid. Test for lactic acid by adding to 5 ml. of gastric contents, 1 ml. of normal hydrochloric acid, and 10 ml. of ether (danger). Shake well and separate the ether in a separatory funnel. Add to the ether 3 ml. of distilled water and a few drops of 10 per cent ferric chloride. Do you obtain a yellow color? Compare with a blank containing the same amount of water and ferric chloride.

96. Occult Blood. Test the gastric contents for occult blood using the benzidine test. Add about 2 ml. of the benzidine reagent and 1 drop of 3 per cent hydrogen peroxide to 1–2 ml. of gastric contents. If hemin is present, a blue color will form.

The benzidine reagent contains 2 g. of benzidine, 10 g. $CH_3COONa \cdot 3\,H_2O$, 10 cc. of glacial acetic acid, 100 ml. of 95 per cent ethyl alcohol, and 100 ml. of water.

URINE

QUALITATIVE ANALYSIS

97. The Detection of Albumin in Urine.

A. *Heat Coagulation Test.* Pour urine suspected of containing albumin into a test tube until tube is about three-quarters full. Hold bottom of tube and heat urine in upper part of tube over a Bunsen flame until urine boils. Now compare upper portion of urine with lower, unboiled portion. Is a precipitate present in the part that has been boiled? The precipitate may be either albumin or tricalcium phosphate. To decide which it is, add 3–4 drops of 10 per cent acetic acid to tube and boil again. Does the precipitate disappear? If it does not, it is albumin. If it does, it is tricalcium phosphate. It may be wise to repeat the addition of the acetic acid and the boiling once more to be sure.

B. *Sulfosalicylic Acid Test.* Place 5 ml. of urine in a test tube. If the urine is not perfectly clear, it is well to filter it. Now add 5 ml. of 10 per cent sulfosalicylic acid and mix well. If a precipitate appears, it is albumin. If albumin is absent, the liquid will remain perfectly clear.

C. *Dinitrosalicylic Acid Test.* Place 5 ml. of urine in a test tube. Filter if not perfectly clear. Now add 10 ml. of saturated solution of dinitrosalicylic acid and mix. A precipitate indicates albumin.

98. Urea—Solubility. Is urea soluble in water? In alcohol?

99. Urea Nitrate and Oxalate. To 2 ml. of concentrated urea solution add 1 ml. of strong nitric acid. To 2 ml. of urea solution add 1 ml. of a saturated solution of oxalic acid. Examine a *small* drop of the urea oxalate crystals under the microscope. Write the structural formulas of urea nitrate and urea oxalate. Draw pictures of urea oxalate crystals.

100. Biuret. Heat a few crystals of urea in a test tube until they fuse, using a free flame. Observe the odor of ammonia. Allow to cool completely, then add 2 ml. of water and 2 ml. of the biuret reagent. Biuret, ammonium cyanate, and cyanuric acid are formed by heating solid urea. Write the structural formulas of these compounds.

101 To a few crystals of urea add 1 ml. of 30 per cent sodium nitrite and 5 ml. of 10 per cent acetic acid. Note the bubbles of gas that are evolved. This is gaseous nitrogen. The acetic acid acting upon the sodium nitrite liberates nitrous acid. The nitrous acid reacts with the urea in the following manner:

$$\begin{array}{l} NH_2 \\ | \\ C{=}O \\ | \\ NH_2 \end{array} + 2\,HONO = CO_2 + 2\,N_2 + 3\,H_2O$$

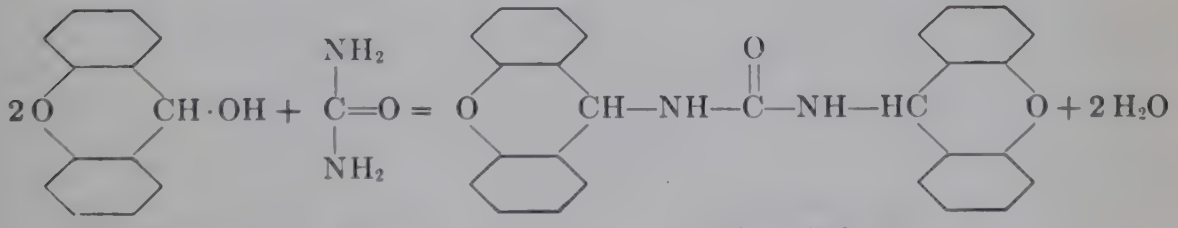
102. Add 2–3 ml. of a solution of freshly prepared sodium hypobromite to a few crystals of urea and observe the evolution of bubbles of gaseous nitrogen. This was the basis of the Knop-Hüfner method for the gasometric estimation of urea in urine. The reaction is usually written:

$$\begin{array}{l} NH_2 \\ | \\ C{=}O \\ | \\ NH_2 \end{array} + 3\,NaOBr + 2\,NaOH = N_2 + Na_2CO_3 + 3\,H_2O + 3\,NaBr$$

Sodium hypobromite is prepared by adding liquid bromine to 20 per cent sodium hydroxide *in the hood.* The reaction is:

$$2\,NaOH + Br_2 = NaOBr^* + H_2O + NaBr$$

103. **Precipitation of Urea by Xanthydrol.** Place 4 ml. of a 0.1 per cent solution of urea in a test tube and add 4 ml. of glacial acetic acid and 10 drops of a 10 per cent solution of xanthydrol in 95 per cent alcohol. Observe the precipitation of dixanthyl urea. The reaction is:



Xanthydrol Urea Dixanthyl urea

This is the basis of the Fosse method for the quantitative determination of urea.

Xanthydrol may be prepared as follows (Holleman, A. F., Organic Syntheses, New York, 7, 88 [1927]): Cover 9 g. of metallic sodium with 20 ml. of dry toluene and melt over a small flame in a pyrex round bottom flask. Add slowly 750 g., or 55 ml. of metallic mercury, having extinguished the flame. Cool, and later pour off the toluene.

* The sodium hypobromite thus formed gradually decomposes forming sodium bromate:

$$3\,NaOBr = NaBrO_3 + 2\,NaBr$$

Suspend 25 g. of xanthone in 175 ml. of 95 per cent ethyl alcohol. Add the mercury amalgam. Shake occasionally. Allow to react for 10 minutes after the solution becomes clear. Decant the solution into 2 liters of cold water. Filter off the precipitated xanthydrol. Wash well with water. According to our own experience, the crystalline material should be dissolved at once, without drying, in 95 per cent ethyl alcohol. If dried, the xanthydrol will be oxidized and ruined. Keep the solution in a brown bottle.

104. Uric Acid—Solubility. Is uric acid soluble in water? In tenth normal sodium hydroxide?

105. Uric Acid—Preparation from Urine. To 200 ml. of urine add 10 ml. of concentrated hydrochloric acid. Mix and allow to stand over night. Observe, under the microscope, the deposit of crystals of uric acid, which are colored brown by uro-erythrin.

106. Folin Reaction. Dissolve some uric acid in 3 or 4 ml. of 10 per cent sodium carbonate solution and add a few drops of the uric acid reagent of Folin and Trimble. Observe the blue reduction compound of phosphotungstic acid.

The uric acid reagent (Folin, O., and Trimble, H., *J. Biol. Chem.* **60**, 473 [1924]) is prepared by placing 100 g. of molybdenum-free sodium tungstate in a flask, adding 33 ml. of 85 per cent phosphoric acid and 150 ml. of water and boiling very gently for an hour. Water should be added from time to time to keep the volume approximately constant. A few pieces of carborundum can be added to prevent bumping. Now add a few ml. of bromine water, or a little bromine vapor in the hood. This decolorizes the blue reduced phosphotungstate, if any is present. Now boil off the bromine, cool, and dilute the reagent to 500 ml. volume.

107. Murexide Test. Place a pinch of uric acid in a porcelain evaporating dish and add 1 or 2 ml. of concentrated nitric acid. Evaporate to dryness on the steam bath *in the hood*. Now add a little ammonium hydroxide. Observe the purple color. With this test the purines, guanine and xanthine give a yellow color, while adenine and hypoxanthine give no color.

108. Silver Urate. Dissolve a trace of uric acid in 1 ml. of tenth normal sodium hydroxide. Add some silver-lactate lactic-acid-solution. Observe the precipitate of silver urate. This is the basis of the Folin isolation method for the estimation of uric acid in blood and urine.

109. Creatinine—Jaffé Test. To 5 ml. of urine add an equal volume of saturated picric acid and about 2 ml. of 10 per cent sodium

hydroxide. Observe the reddish color. This is the basis of the Fólin method for the quantitative estimation of creatinine. According to Greenwald, the colored compound is a tautomeric form of sodium creatinine picrate. Heat some of the colored liquid to boiling in a test tube. Notice that the color changes from reddish orange to brown. The brown material is picramic acid which is formed by the reduction of the picric acid.

110. Indican—Obermayer's Test. To 5 ml. of urine add an equal volume of Obermayer's reagent. Add 2 ml. of chloroform and mix thoroughly. The ferric chloride serves as the oxidizing agent. The blue substance is indigo. Write the reaction.

Note: If the chloroform becomes emulsified by mixing it will at first appear white, but as the droplets coalesce, the whiteness will pass, and the blue color, if present, will become visible. Most samples of normal urine contain little or no indican.

Obermayer's reagent is made by dissolving 3 g. of ferric chloride in 1 liter of concentrated hydrochloric acid.

111. Phosphates. To 5 ml. of urine add 1 or 2 ml. of ammonium hydroxide. Mix. Observe the precipitate of amorphous tricalcium phosphate, $Ca_3(PO_4)_2$. Alkali phosphates remain in solution. Magnesium ammonium phosphate, $MgNH_4PO_4$, may crystallize out on standing.

112. Pus and Casts in Urine. Centrifuge about 10 ml. of fresh, normal urine in a 15 ml. centrifuge tube (first having carefully read the directions concerning use of the centrifuge on page 12). Place a very small portion of the precipitate on a slide, cover with a cover glass, and observe under the microscope. Note that pus cells and casts are not present.

Examine under the microscope a drop of a special sample of urine which contains pus cells and casts.

Test the pus cells centrifuged from 10 ml. of urine by adding 2 ml. of water, 2 drops of fresh 2 per cent alcoholic gum guaiac, and 1 drop of 3 per cent hydrogen peroxide. A blue color will be produced, owing to the presence of peroxidase in the pus cells.

Note: This experiment is usually given as a demonstration.

QUANTITATIVE ANALYSIS

Note: Be sure you are familiar with instructions on pages 5–14 before starting these experiments.

113. Kjeldahl Method for Total Nitrogen. Pipette exactly 5 ml. of urine into a clean 500 ml. Kjeldahl flask, *add from a graduate* 20 ml.

of special concentrated sulfuric acid and about 20 drops of saturated copper sulfate solution (*danger*). Heat in the digestion rack until the yellow color is all gone and the liquid is a greenish blue. Turn the flask if the carbonaceous material has not been washed down by the hot acid. The Bunsen flame must never be turned on so high that it strikes above the meniscus of the acid. Now heat for 40 minutes more and allow to cool in the rack. Do not remove from the rack while fumes are being given off. In fact, it is necessary to wait until the contents of the flask have become well cooled. This will take 20 minutes after the flame has been turned off.

Now, after the solution has been well cooled (*otherwise, there will be an explosion*), carefully add 300 ml. of distilled water. Mix very thoroughly and add about a quarter teaspoonful of coarse carborundum. It will be well to cool the contents of the flask at this point in order to avoid violent boiling at a later stage. Violent boiling is certain to occur if one has used much less than 300 ml. of distilled water to dilute the acid, or if one has used considerably more than 25 ml. of concentrated sulfuric acid for the digestion.

Run into a clean large Erlenmeyer flask 60 ml. of tenth normal hydrochloric acid and add 4–6 drops of Methyl red. Now pour down the side of the inclined Kjeldahl flask 60 ml. of saturated sodium hydroxide. *Do not mix.* Adjust the Folin distillation apparatus (see note) under the supervision of the instructor, light the micro-burner, place under the flask, and *at once rotate the flask until the contents are well mixed.* If the material is not mixed up before heating, the analysis will blow up. Heat very cautiously until the boiling is well under way. After boiling has continued for 30 minutes, raise the flask so that the delivery tube is out of the acid. Continue heating until the bumping begins. Allow the acid solution to cool and titrate to an orange shade with tenth normal sodium hydroxide.

Calculate the total nitrogen in the 24-hour sample of urine.

Note: This apparatus of Folin is a substitute for the regulation Kjeldahl still. It is composed of a wide-bore delivery tube which is attached by a rubber tube to a distillation tube. The distillation tube has a number 6 rubber stopper at one end and a side hole for the entrance of steam.

114. Micro-Kjeldahl. Pipette 5 ml. of blood filtrate (Folin-Wu), or 1 ml. of urine (diluted 1–10 or 1–20), or a suitable volume of any solution you desire to analyze, into a thin-walled Pyrex digestion tube (25 × 200 mm.). Add 1 ml. of ammonia-free concentrated sulfuric

acid and 1 drop of 5 per cent copper sulfate. Add also a piece of Aloxite or carborundum to prevent bumping. Mix and heat *in the hood* over a micro-burner. Have the flame small and the bottom of the test tube only about 0.5 to 1.0 cm. above the burner. After heating until white fumes of sulfur trioxide come off, cool, add 1 drop of 30 per cent hydrogen peroxide and heat again. Repeat, adding in all some 5 or 6 drops of hydrogen peroxide, or until the brown or yellow color of the digest has completely disappeared. Now allow to cool for 10 minutes. Do not remove from the hood. Add 5 ml. of distilled water and mix.

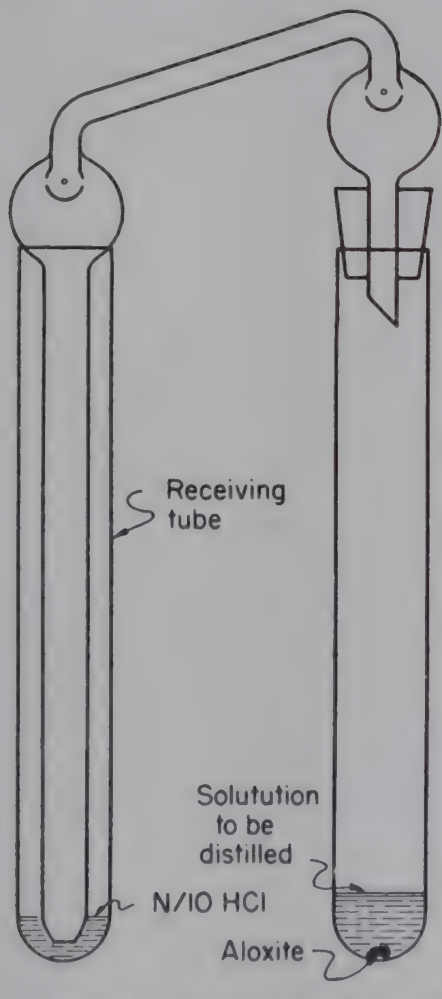


FIG. 11. Distillation apparatus for micro-Kjeldahl.

The ammonia in the digest is now removed either by aerating or by distilling after setting it free through the addition of alkali.

Distillation: Set up the apparatus with special traps according to directions given by the instructor (see Fig. 11). Add a piece of paraffin to the digestion tube to prevent foaming. Use a large (25 × 200 mm.) tube graduated at 50 ml. for receiver. To this receiving tube, add 3 ml.

of $N/10$ HCl. When all is ready for distillation, 3 ml. (approximately) of 70 per cent NaOH is added to the digestion tube, the distillation tube is quickly put in place, and the ammonia is distilled into the acid using a micro-burner, slowly for two minutes, and more rapidly for three minutes. Then the receiving tube is lowered and distillation is continued for three minutes longer to rinse out the distillation tube.

If the quantity of ammonia in the analysis is not much more than 0.10 mg. the solution is cooled and then diluted to about 35 ml. and mixed well. One now rotates, or swirls, the solution and adds quickly 2.5 ml. of Nessler solution. The solution is diluted to exactly 50 ml. and mixed. The photoelectric colorimeter (Klett-Summerson) is set at the null point, using a solution containing 5 ml. of Nessler solution diluted to 100 cc. with distilled water and with a blue glass filter. The unknown solution is now read. The reading is referred to a standard graph and the mg. of nitrogen obtained.

If the quantity of ammonia in the analysis is between 0.2 and 0.5 mg. the solution is poured into a 200 ml. volumetric flask, using a clean funnel, and water is used to wash in the rest. The flask is filled two-thirds full and swirled. One adds quickly 10 ml. of Nessler solution, dilutes to the 200 ml. mark, mixes, and reads in the photoelectric colorimeter, using the blue filter.

The standard graph is prepared by Nesslerizing ammonium sulfate solutions, containing 0.1, 0.2, 0.4, and 0.7 mg. of nitrogen, in 200 ml. flasks, diluting to volume, mixing and reading in the colorimeter. One then plots mg. of nitrogen against colorimeter readings. Before making the readings the colorimeter is set at the null point by employing a solution containing 10 ml. of Nessler solution diluted to 200 ml. volume.

The ammonium sulfate solution should be prepared from purest ammonium sulfate that has been dried in a desiccator. The ammonium sulfate concentration can be 471.7 mg. per liter, giving a concentration of 0.1 mg. of nitrogen per ml.

Nesslerized solutions should be water-clear. If properly prepared they do not increase in color upon standing, as has been stated in the literature. Nessler solution should be kept in pyrex bottles or carboys. Do not use glass-stoppered containers for storing Nessler solution.

The Folin-Wu modified Nessler solution is prepared as follows: Place 150 g. of potassium iodide, 110 g. of iodine, 100 ml. of water, and 140–150 grams of metallic mercury in a flask and rotate until practically all of the iodine has been used up. When the last of the iodine

is being used up, cool the flask in running water. Pour and wash off the potassio-mercuric iodide and dilute to a volume of 2 liters. To 3500 ml. of 10 per cent sodium hydroxide add 750 ml. of the potassio-mercuric iodide and 750 ml. of distilled water.

This Nessler solution should be kept in a pyrex bottle or carboy. If kept in ordinary glass it will dissolve enough electrolytes to become unstable when used for Nesslerization.

115. Inorganic Phosphates. (Sumner, J. B., *Science* **100**, 413 [1944]). Pipette into a 25 × 200 mm. tube, graduated at 50 ml., a known volume of the orthophosphate solution to be analyzed. The amount of phosphorus present should lie between 0.01 and 2.2 mg. Add 5 ml. of 6.6 per cent ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$, and distilled water to nearly 40 ml. Add 5 ml. of 7.5 *N* sulfuric acid and mix gently by rotating the tube. Now add 5 ml. of the ferrous sulfate solution (4 g. $FeSO_4.7H_2O$ in 50 ml. of water and 1 ml. of 7.5 *N* H_2SO_4). Dilute to the 50 ml. mark, stopper with a clean rubber stopper and invert the tube four or five times. Prepare a blank in a second tube, using the same amount of molybdate, sulfuric acid, and ferrous sulfate. Now employ the blank solution to set the colorimeter at the null point using the red glass filter (Klett-Summerson or Fisher colorimeter). Next read the unknown. The amount of phosphorus present is obtained from a graph. This is prepared by running determinations of various quantities of a standard solution of pure KH_2PO_4 (see Exp. 49). The graph obtained will be a perfectly straight line.

116. Total Phosphorus. Pipette an aliquot of the solution into a thin-walled 25 × 200 mm. tube. Add 5 ml. of 7.5 *N* sulfuric acid and heat with a micro-burner until white fumes appear. Allow to cool and add 1 or 2 drops of redistilled 30 per cent hydrogen peroxide. (Thirty per cent hydrogen peroxide can be distilled *in vacuo* using the ordinary distilling flask and Liebig condenser.) Heat again. Again allow to cool, add hydrogen peroxide and heat, continuing until the solution is perfectly free from brown or yellow color. Now cool and add 5 ml. of distilled water and a glass bead. Heat until white fumes appear. This will drive off all of the hydrogen peroxide.

Now cool, add water until the volume is 35 to 40 ml., add 5 ml. of the 6.6 per cent molybdate, mix and add 4 ml. of the ferrous sulfate, continuing as is described in Exp. 115. Note that it is absolutely necessary to remove all of the hydrogen peroxide or values for phosphorus will be low.

117. Quantitative Determination of Acetone in Urine (Shaffer, P. A., and Marriott, *J. Biol. Chem.* 16, 265 [1915]). First, the urine is distilled from acid, then the distillate is redistilled from alkali to separate the acetone from interfering substances.

Second, the acetone in the second distillate is treated with excess of iodine and sodium hydroxide. Some of the iodine is used up by the formation of iodoform. After 5 minutes the solution is made acid and the remaining iodine titrated with sodium thiosulfate, using starch as indicator.

Procedure: Pipette 5 ml. of acetone solution containing about 25 mg. of acetone, into a 500 ml. Erlenmeyer flask and add to this exactly 25 ml. of standard iodine solution and about 20 ml. of 10 per cent sodium hydroxide. Shake and allow to stand for 5 minutes. Add about 9 ml. of concentrated hydrochloric acid and titrate at once with standard sodium thiosulfate until the color of the iodine becomes yellow. Now add 1 ml. of starch solution and titrate until the blue color has nearly disappeared. Record the number of ml. of sodium thiosulfate solution needed for the back-titration.

Calculation: Calculate the number of ml. of $N/10$ iodine solution which was equivalent to the acetone present. Knowing that 1 ml. of $N/10$ iodine is equivalent to 0.968 mg. of acetone, compute the number of mg. of acetone in 5 ml. of the unknown. Enter this figure in your notes and report it to the instructor.

Preparation of Reagents: The iodine solution is prepared by weighing out about 13 g. of iodine and dissolving this in 150 ml. of water containing about 18 g. of potassium iodide. When the iodine has all dissolved, it is diluted to 1000 ml. in a volumetric flask fitted with a glass stopper, and is well mixed. This iodine solution should be kept in the dark, or else should be placed in a brown bottle.

The sodium thiosulfate solution is prepared by dissolving about 25 g. of sodium thiosulfate in water, diluting to 1000 ml. volume and mixing. It is best to employ freshly boiled distilled water which has afterwards been cooled in a flask fitted with a soda-lime tube to keep out carbon dioxide.

The thiosulfate solution is standardized as follows: Weigh accurately from a weighing bottle into an Erlenmeyer flask 0.1 to 0.2 g. of resublimed iodine which has been well dried in a desiccator containing calcium chloride. Add at once about 1 g. of purest potassium iodide and about 20 ml. of water. Mix gently until the iodide has dissolved

and titrate with the thiosulfate until most of the iodine has been used up. Now add the boiled starch solution and titrate to a faint blue color. Knowing the weight of pure iodine employed, calculate the normality of the thiosulfate solution.

The iodine solution is now standardized against the thiosulfate solution.

In place of resublimed iodine to standardize the thiosulfate, one can use pure, dry potassium iodate, 3.567 g. of which, when made up to 1 liter volume with water, is tenth normal. One pipettes 25 ml. of iodate into a flask, adds water, 1 to 2 g. of potassium iodide (iodate-free) and 5 ml. of 1 *N* sulfuric acid. This is then titrated using starch and the thiosulfate.

BLOOD: QUANTITATIVE ANALYSIS

Drawing Blood. Specimens of blood from human beings are usually obtained from the median basilic vein in the arm. The skin is first rubbed with a swab of cotton moistened with alcohol for the purpose of disinfection. A rubber tube is tied tighly about the upper arm to cause the veins of the arm to fill with blood, and the subject is asked to clench the fist several times. A hollow needle, No. 18, is used. It should be run through with a wire to make certain that it is not stopped up, and should be sterilized by heating in boiling water for 5 minutes. The vein is held between the thumb and index finger, and the needle is stuck into the vein, being pointed towards the shoulder. A flask containing the requisite amount of dry potassium oxalate is held under the large end of the needle to catch the blood. The operator must take care to secure approximately the amount of blood for which the amount of oxalate has been weighed out (20 mg. of potassium oxalate are ample for 10 ml. of blood). As soon as blood begins to flow from the needle, the rubber tourniquet must be removed; otherwise a hemorrhage under the skin will occur when the needle is removed. As the blood drips into the receiver, the latter must be rotated to mix the blood with the oxalate. When sufficient blood has been collected, press a swab of cotton moistened with tincture of iodine against the skin above the needle, withdraw the needle and press the swab firmly against the wound and hold it there for about a

minute.

118. Preparation of Protein-free Blood Filtrate (Folin, O., and Wu, H., *J. Biol. Chem.* **38**, 81 [1919]). Pipette exactly 10 ml. of oxalated blood onto a clean, dry 250 ml. Erlenmeyer flask. Add 70 ml. of distilled water and mix. This lakes the corpuscles. Add 10 ml. of 10 per cent sodium tungstate solution and mix. Now add slowly, with constant rotation, 10 ml. of two-thirds normal sulfuric acid. Stopper with a clean rubber stopper and shake well. It will give a metallic click if the protein has been properly precipitated. Allow to stand for 20 minutes and filter through a dry filter paper, on a dry funnel, into a dry flask. Keep the funnel covered with a watch glass to prevent evaporation. It is best to add only a few cubic centimeters at first, until the filter paper is wet, and then more may be added.

The filtrate which is obtained contains the blood constituents (urea, uric acid, glucose, creatinine, amino acids, etc.) ten times diluted. The filtrate must be water-clear and must not be acid to Congo red paper.

The sodium tungstate used ($Na_2WoO_4 \cdot 2\ H_2O$) should not be acid, *i.e.*, paratungstate. If it is acid, it should be made neutral to phenolphthalein before making the 10 per cent sodium tungstate solution. This can be accomplished by titrating with sodium carbonate solution.

The oxalated blood should be analyzed soon after it has been drawn. Even when kept on ice the blood sugar slowly disappears.

119. Urea Nitrogen. *Folin-Sumner* (unpublished). Rinse a test tube, graduated at 25 ml. with alkaline 60 per cent potassium iodide solution to remove absorbed mercury. Rinse with tap water and finally with distilled water. Pipette into the tube exactly 5 ml. of blood filtrate. *This must not be acid to Congo red paper.* Add 3 drops of citrate buffer, pH 6.1, and mix. Add 1 sq. cm. of urease paper, mix, and place the tube in water at 40–50° C. for 20 minutes. Now remove the tube and cool in running water. Add 10 drops of 10 per cent Rochelle salt and dilute to 18–20 ml. Now, while rotating the tube, add 1.2 to 1.3 ml. of Nessler solution. Dilute to the 25 ml. mark, mix, and read the solution in the colorimeter as in Exp. 114. Determine the amount of urea nitrogen present by reference to the standard graph.

The color of the unknown will be yellow on account of the effect of creatinine, creatine, etc. on the Nessler solution, but this will not greatly interfere with the accuracy of the result. The Rochelle salt which is added prevents the Nesslerization from becoming turbid.

If the nephritic blood filtrate is used, it may be necessary to repeat the analysis, using, instead of 5 ml. of blood filtrate, only 2 or even 1 ml.

The citrate buffer is prepared by mixing 21 ml. of 0.5 *M* citric acid with 500 ml. of 0.5 *M* trisodium citrate.

120. Blood Urea Nitrogen by Distillation (Folin, O., and Svedberg, A., *J. Biol. Chem.* **88**, 77 [1930]). Pipette 5 ml. of blood filtrate into a large thin-walled test tube. Add 2 drops of citrate buffer, pH 6.1, and a piece of urease paper about 2 cm. long and 1 cm. wide. Place the tube in water at 45° C. and allow it to remain for at least 10 minutes, mixing occasionally. Cool the tube and add an anti-bumping tube (instead of using an anti-bumping tube, one can use a piece of carborundum), 2 drops of anti-foaming mixture, and 2 ml. of saturated borax solution. Connect at once with a distillation apparatus composed of a rubber stopper fitted with a bent 5 ml. pipette that has had the constricted tip cut off. Clamp the tube firmly about 1 cm. above a microburner. Now distill into a large test tube which is graduated at the 25 ml. mark and which contains 1 ml. of 0.1 *N* hydrochloric acid. Have the end of the delivery tube immersed in the acid. Boil gently for the first minute and briskly for the next three minutes. Then raise the delivery tube out of the acid and boil for 1 minute more.

Instead of using the distillation apparatus described here, you may use the same apparatus which was used for distillation in the determination of total nitrogen by the micro-Kjeldahl method.

Cool the distillate, dilute to about 20 ml., mix well and add 1.2 to 1.3 ml. of Nessler solution. Dilute to the 25 ml. mark, mix and read in the photoelectric colorimeter. Obtain the mg. of urea nitrogen by referring to the standard graph (see Exp. 114).

The urease paper may be prepared as follows: To 70 ml. of 9.6 per cent neutral phosphate buffer, add 120 ml. of water and 145 ml. of 95 per cent ethyl alcohol. Dilute to 350 ml. and mix with 100 g. of jack bean meal. Filter. This will take about 2 hours. To every 100 ml. of filtrate, add 1 g. of neutralized cysteine hydrochloride. Pass strips of filter paper (Schleicher and Schüll, No. 595) through the solution and hang them up to dry in a room free from fumes of acid or ammonia. Later, cut the strips so that they are 1 cm. wide. One square centimeter of this paper should contain about 0.35 units of urease.

Anti-bumping tubes can be made by sealing about one-half inch lengths of glass tubing to glass rods. The diameters should be about 6 to 8 mm.

The anti-foaming mixture is crude fuel oil, 1 part; toluene, 10 parts.

121. Blood Sugar. *Folin-Wu.* Pipette 2 ml. of blood filtrate into a clean Folin-Wu sugar tube. Add 2 ml. of the Folin-Wu copper reagent. Mix and heat in boiling water 6 to 8 minutes. Remove and at once add 2 ml. of the phosphomolybdic acid solution. Let stand for 2 minutes. Cool, dilute to the 25 ml. mark and mix. Using a red filter and a blank containing 2 ml. of copper reagent and 2 ml. of phosphomolybdic acid diluted to 25 ml., set the photoelectric colorimeter at the null point. Now read the unknown solution. From the graph find out how many mg. of glucose are present.

The graph is prepared by running determinations on standard glucose solutions containing 0.1, 0.2, and 0.4 mg. of glucose and plotting the readings against mg. of glucose on suitable coordinate paper.

This method depends upon the reduction of a modified Fehling's solution (which is very weakly alkaline) by heating with the sugar of the blood filtrate. Upon adding the phosphomolybdic acid this is reduced by the cuprous oxide to produce the deep blue "reduced phosphomolybdic acid." The phosphomolybdic acid solution contains a small amount of phosphotungstic acid to give the right shade of color.

The Folin-Wu copper reagent is made by dissolving 40 g. of anhydrous sodium carbonate in 400 ml. of water. This is filtered into a liter volumetric flask. Add 7.5 g. of tartaric acid. When this is dissolved, add 4.5 g. of crystallized copper sulfate. When dissolved, dilute to 1 liter volume and mix. If a sediment forms on standing, decant.

The phosphomolybdic acid reagent is prepared by placing 35 g. of molybdic acid and 5 g. of sodium tungstate in a liter beaker. Now add 200 ml. of 10 per cent sodium hydroxide and 200 ml. of water. Boil vigorously for from 20 to 40 minutes to remove the ammonia. Cool, dilute to 350 ml. and add 125 ml. of 85 per cent phosphoric acid. Dilute to 500 ml. and mix (Folin, O., and Wu, H., *J. Biol. Chem.* **41**, 367 [1920]; Folin, O., *J. Biol. Chem.* **82**, 83 [1929]).

122. Blood Sugar. Method of Somogyi, Shaffer and Hartmann (Somogyi, M., *J. Biol. Chem.* **87**, 339 [1930]). To one volume of oxalated blood add 7 volumes of water and mix to lake. Add 1 volume of 10 per cent $ZnSO_4 \cdot 7H_2O$ and mix. With constant rotation add 1 volume of 0.5 *N* sodium hydroxide. Stopper the flask and shake it well. Filter through dry filter paper on a dry funnel into a dry flask. Employ 5 ml. of the filtrate for the determination of sugar, following the directions already given on page 39. From the volume of thiosulfate obtained upon substracting the amount used up by the unknown from the amount used up by the blank one can read the mg. of glucose in the aliquot of

the filtrate in the table on page 41. Calculate the mg. of glucose in 100 ml. of blood.

123. The Electrometric Determination of pH. Various methods are used for determining the pH of solutions. Formerly indicators were commonly used, but now these have been very largely replaced by electrometric methods, except for rough approximation. If an approximate value is sufficient, indicators are frequently more convenient than electrometric methods. In recent years various convenient kinds of electrometric apparatus for determining pH have appeared on the market. A few years ago most of these made use of the quinhydrone electrode. Recently the glass electrode has become very popular. This electrode has many advantages over the quinhydrone electrode, which accounts for its popularity.

In all electrometric measurements of pH the same general principles are used:

1. Some sort of electrical cell is set up so that the difference in potential between the electrodes of the cell is a function of hydrogen ion activity, (H^+).*

2. The potential difference, E, between the electrodes is measured by means of a potentiometer circuit. A diagram of such a circuit is shown in Fig. 12.

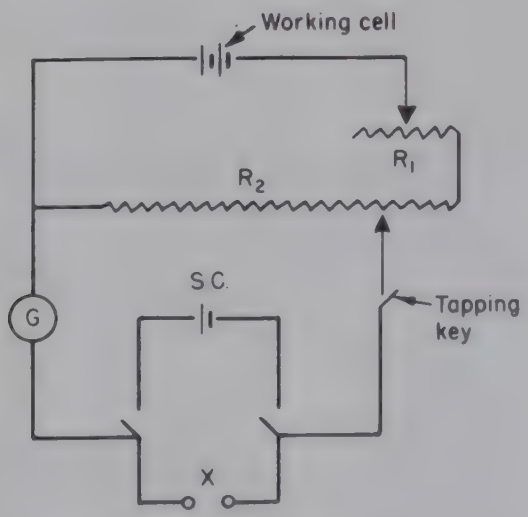


Fig. 12. Potentiometer circuit.

To use such a potentiometer circuit:

1. Connect the standard cell, S.C., into the circuit with the uniform resistor R_2, set at the proper value. On modern instruments, the setting

*In aqueous solutions the hydrogen ions are hydrated so that they should be written as H_3O^+, or some other hydrated ion, but for the sake of convenience they will be represented as H^+.

of R_2 is ordinarily done automatically by the same switch which connects the standard cell into the circuit.

2. Adjust the resistor R_1 until there is no deflection of the galvanometer when the tapping key is closed momentarily. This sets the scale of the uniform resistor, *e.g.*, a slide wire, so that the divisions on it read directly in volts. (With some instruments the readings are directly in pH.)

3. Connect the unknown cell into the circuit in place of the standard cell and adjust R_2 until there is again no deflection of the galvanometer when the tapping key is closed momentarily. The reading of the scale now gives the voltage of the unknown cell, X, or, in some cases, the pH.

Check the circuit periodically against the standard cell so that you will avoid errors due to a change in the voltage of the working cell.

A *quinhydrone* cell (see Fig. 13.) can be used as follows to determine the pH of an unknown solution:

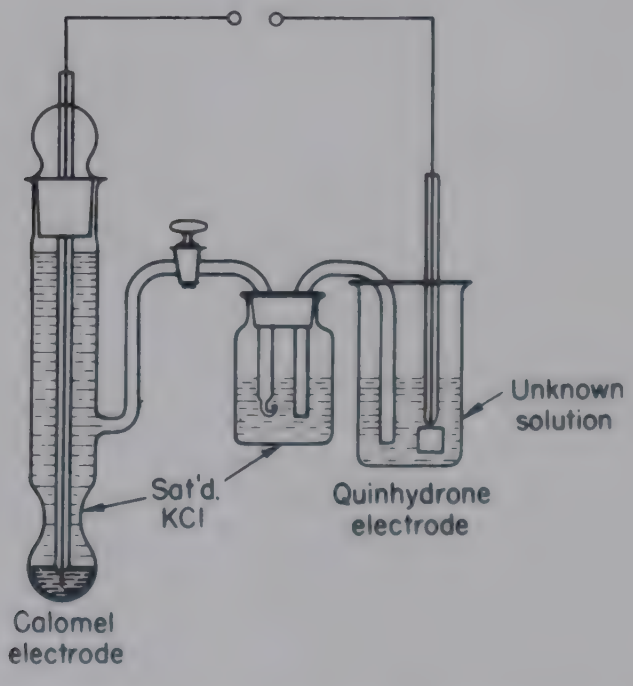


Fig. 13. Quinhydrone cell for pH determination.

1. Open the stopcock on the side arm of the calomel electrode and make sure there are no bubbles in the side arm of the electrode vessel.

2. Rinse the quinhydrone electrode vessel thoroughly with the solution to be measured, or else clean it and allow to dry.

3. Rinse the platinum electrode with distilled water and then with the solution to be measured.

4. Pour some of the solution into the quinhydrone electrode vessel,

add a small amount of solid quinhydrone and suspend the latter by shaking the vessel.

5. Place the platinum electrode in the solution and connect the solution with the vessel of KCl solution by means of an agar-KCl bridge (3 per cent agar saturated with KCl). Connect the platinum electrode to the circuit.

6. Measure the voltage of the quinhydrone cell as outlined above.

7. Take the temperature of the solution.

8. Rinse the quinhydrone electrode vessel with distilled water. Rinse the platinum electrode with distilled water and place it in the vessel of distilled water provided. Turn off the switches. Close the stopcock on the calomel electrode.

The pH of the unknown solution can be calculated using the following equation:

$$\text{pH} = \frac{0.453 - \text{E}_{\text{cell}}}{1.9837\text{T} \times 10^{-4}}$$

where E_{cell} is the voltage measured for the cell, when the cell is composed of a saturated calomel electrode and a quinhydrone electrode. T is the temperature (absolute).

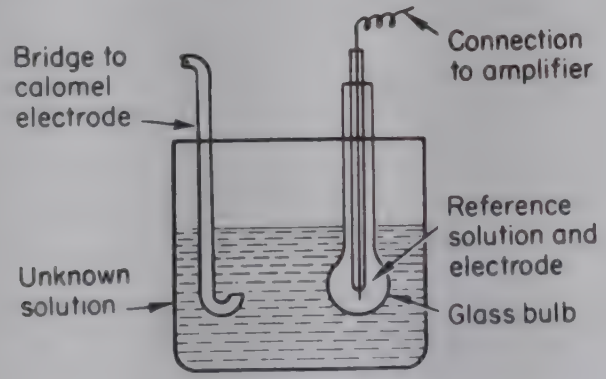


Fig. 14. Glass electrode.

The use of a *glass electrode* to determine the pH of an unknown solution requires a modification of the potentiometer circuit shown in Fig. 12. The glass electrode, as ordinarily used, is essentially a glass bulb containing a reference solution and an electrode. The electrode inside the glass bulb can be a quinhydrone electrode, although ordinarily a more stable electrode, such as a silver-silver chloride electrode, is used (see Fig. 14). The glass bulb is immersed in the unknown solution. The difference in hydrogen ion activity between the reference solution and unknown solution creates a difference in potential between the

inside and outside of the bulb. It is this difference in potential that is measured and used to calculate the pH of the unknown. Since the resistance of the glass bulb is very high it requires a very sensitive apparatus to measure the potential difference between the unknown and reference solutions. Hence the circuit shown in Fig. 12 has to be modified, and ordinarily it is modified by including some electronic arrangement for amplifying the current so that the required potential difference can be easily and accurately measured.

The pH of an unknown solution may be measured using a glass electrode in the following way. (These directions are for the Beckman pH meter — Laboratory Model G. With small changes, they will apply to other instruments.)

1. Set the range switch to "pH" and move the operating switch lever to the vertical position. After a few seconds, adjust the milliammeter needle to zero by rotating control No. 1.

2. Set the temperature compensator to the temperature of the unknown solution. Check the circuit against the standard cell by holding the operating switch lever in position No. 2 and adjust the milliammeter needle to zero by means of control No. 2. Return the operating switch lever to the vertical position. The milliammeter needle should still read zero. Repeat this checking until the milliammeter needle reads the same with operating switch lever in position No. 2 and vertical. This check must be repeated each time the temperature compensator is changed; otherwise only infrequent checking is required.

3. Open the door in front of the instrument and carefully lower the beaker from the electrodes. Rinse the beaker and electrodes with distilled water. Partially fill the beaker with some of the unknown solution and carefully rinse the electrodes by raising the beaker around them. Discard this rinsing. Place some more of the unknown solution in the beaker and again raise the beaker around the electrodes and close the door.

4. Push down the button in the center of the slide wire knob and rotate the knob until the needle of the milliammeter comes to zero. Release the button. If the needle fails to remain at zero readjust control No. 1 and repeat the operation. The scale now reads the pH at the temperature of measurement.

5. Carefully rinse the electrodes and beaker once more and leave the electrodes immersed in distilled water in the beaker.

6. Close the cover, unless someone else is going to use the instrument immediately. Closing the cover automatically turns the operating switch lever to the "off "position.

If you desire to check the pH apparatus to tell whether or not it is working properly, test the pH of a solution of 0.05 *M* potassium acid phthalate. This has a pH of 4.00 at 20° C. and its pH differs from this by only a negligible amount over a temperature range from about 18 to 30° C. The glass electrode pH meter is standardized against this solution by means of a zero adjustor. Any changes in the zero adjustor should be made *only by the instructor*.

Determine the pH of the unknowns which are provided and report the results to the instructor. You may determine the pH of one of the buffer solutions in the laboratory as a preliminary, if you wish, to acquaint yourself with the method.

If you are not familiar with the preparation of buffer solutions, you should make one or more buffer solutions and check their pH. See Gortner (Outlines of Biochemistry, 2d ed., Wiley and Sons, pp. 117–126 [1938]), or Clark (cited below) for details.

Additional information on pH measurements will be found in Clark, W. M., The Determination of Hydrogen Ions, 3d ed., Williams and Wilkins (1928); Dole, M., The Glass Electrode, John Wiley and Sons Company (1941); Britton, H. T. S., Hydrogen Ions, 3d ed., Van Nostrand (1943).

Part II

AMINO ACIDS

124. Preparation of Tyrosine. 500 g. of silk waste has been boiled in the hood with 1 liter of concentrated hydrochloric acid until abiuret (10–15 hours), using a reflux condenser.

To precipitate the tyrosine it is best to bring the material to pH 5.6, the isoelectric point of tyrosine. This has been done already by the instructor by adding alkali and testing with the glass electrode.

Shake the material well, take 15 ml. and filter off the tyrosine. Wash with a little water. Now dissolve in 25 ml. of boiling water, adding filter paper and all. Add 1 teaspoonful of bone black and boil for 1 or 2 minutes. Filter hot. The filtrate must come out clear. The tyrosine will separate upon standing overnight. Examine a small drop of water suspension of the crystals of tyrosine under the microscope. Filter off the tyrosine, dry, and place in a labelled test tube. Give the preparation to the instructor after you have finished with it.

125. Test for Tyrosine with Phenol Reagent. Dissolve a trace of tyrosine, prepared in experiment 124, in 1 ml. of a 20 per cent sodium carbonate solution and add about 4 ml. of water. Now add a few drops of the phenol reagent of Folin and Denis. Notice the blue color. This is due to the reduction of the phosphotungstic and phosphomolybdic acids by the tyrosine.

The "phenol reagent" (modification of Folin, O., and Ciocalteu, *J. Biol. Chem.* **73**, 627 [1929]), which is a mixture of phosphotungstic and phosphomolybdic acids, is made as follows: Place in a 1500 ml. flask 750 ml. of water, 100 g. of sodium tungstate, 25 g. of sodium molybdate, 50 ml. of 85 per cent phosphoric acid, and 100 ml. of concentrated hydrochloric acid. Boil gently for 10 hours using a watch glass and funnel to help condense the vapors. Now add 150 g. of lithium sulfate, 50 ml. water, and a few drops of bromine. Boil off the bromine and dilute to 1 liter volume.

126. Millon's Test. Boil a trace of tyrosine prepared in experiment 124 with 3 ml. of water and a few drops of Millon's reagent. This is a general test for phenols.

Millon's reagent can be prepared by adding 60 g. of red mercuric oxide to 45 ml. of concentrated nitric acid and 50 ml. of water. When

the oxide has dissolved, add 50 ml. of 30 per cent sodium nitrite and mix. Millon's reagent contains mercuric nitrate and mercuric nitrite.

127. Cystine. Cystine is prepared by boiling wool or hair with concentrated hydrochloric acid for 8 to 10 hours. Place the hot solution in the hood, since vapors of HCl are given off, and add a hot saturated solution of sodium acetate until all of the hydrochloric acid has been neutralized. This point is detected by testing with Congo red paper. Cystine crystallizes out slowly. It can be purified by dissolving in dilute hydrochloric acid, boiling with bone black, in the hood, filtering hot, and repeating the addition of sodium acetate. Ask the instructor to show you crystals of cystine under the microscope, if you do not prepare cystine yourself.

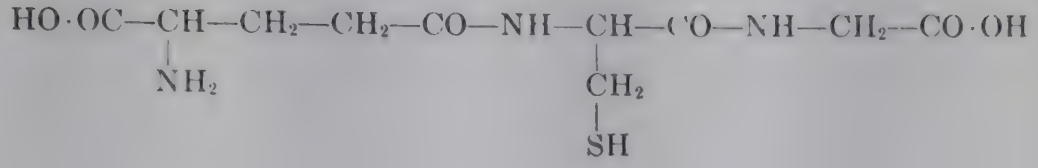
To 1 ml. of cystine solution in hydrochloric acid, add 1 ml. of 20 per cent sodium carbonate and 1 ml. of the uric acid reagent of Folin and Trimble (phosphotungstic acid). Now repeat, adding to the cystine solution 1 ml. of 20 per cent sodium carbonate, 2 ml. of 10 per cent sodium sulfite, and, after 5 minutes, 1 ml. of the uric acid reagent. The sulfite reduces the cystine to cysteine and the salt of cysteine sulfonic acid (Clark, H. T., *J. Biol. Chem.* **97**, 235 [1932]). The former substance, in the presence of sodium carbonate, reduces the uric acid reagent. Cystine itself has no such action. Write the structural formulas of cystine and of cysteine.

128. Unoxidized Sulfur. Boil some of the cystine solution with concentrated sodium hydroxide and a few drops of lead acetate solution. Lead sulfide is formed.

129. Demonstration — Determination of Amino Nitrogen by Van Slyke Method (Van Slyke, D. D., *J. Biol. Chem.* **12**, 275 [1912]; **16**, 121, 125 [1913]; **83**, 425 [1929]). This demonstration should be fully written up by the student and a diagram of the apparatus should be drawn in the note book. For what purpose is amino nitrogen determined?

Note: Primary amino groups give off gaseous nitrogen when acted on by nitrous acid. Secondary and tertiary amino groups do not.

130. Glutathione. To about 5 ml. of ground liver, or fresh yeast suspension add 4–5 drops of a fresh solution of nitro-prusside, about 2 g. of solid ammonium sulfate, and 3 ml. of 10 per cent acetic acid, and shake. Now pour on an excess of ammonia. Note the pink color. This is a test for glutathione, the tripeptide which was discovered in yeast by F. G. Hopkins. Glutathione is glutamyl-cysteinyl-glycine and its structural formula is:



PROTEINS

131. Make a protein solution by mixing about 2 g. of egg albumin powder with 100 ml. of water. Filter and use the filtrate for the following experiments. The egg albumin powder is simply desiccated egg white. It contains the proteins ovalbumin and conalbumin, which constitute about 90 per cent of protein matter present. The remaining 10 per cent of protein material is composed of ovoglobulin, ovomucin, and ovomucoid. A consid[illegible] amount of the protein in the preparation will be found to [illegible]um hydroxi[illegible] account of having been denatured during the proc[illegible]on with 1 drop of 1 p[illegible] [illegible]0 drops of 10 per cent acetic

132. Biuret Test. To 2 ml. of [illegible] the heat coagulation of "egg about 2 ml. of biuret reagent and [illegible] Compare with a blank. The biur[illegible] with 1 drop of 1 per cent acetic is given by the amide and imi[illegible]oride. Salt causes the denatured The development of color in th[illegible] good reagent for the acidification of The test is more delicate thar[illegible] heat coagulation is composed of sodium test more certain (when onl[illegible]ic acid 1 per cent. This is a "buffer" run a blank together with [illegible]y constant. One milliliter of this buffer same volume of liquid (wa[illegible]e. same number of drops [illegible]. **Between Denaturation and Coagulation.** after 5 minutes compar[illegible]f salt-free ovalbumin in boiling water for 5 paper by daylight. If [illegible] the solution has become slightly opalescent, in color while the blan[illegible]ed. Now add some sodium chloride solution is present in the unk[illegible]cured albumin is precipitated. Heating salt-free as the yellow color c[illegible] and changes it from a lyophilic to a lyophobic

Biuret reagent [illegible]es of the latter are kept from uniting because qualitative sugar r[illegible] charges. These charges are neutralized by the If there is any fe[illegible]-free ovalbumin can be prepared by dialyzing a to settle out; afte[illegible]lline ovalbumin in a collodion membrane against

133. Xantho several days. The distilled water is changed each with 1 ml. of [illegible] is used as a preservative. If an electro-dialyzer is ammonium h[illegible]n be removed within a few hours. compounds r[illegible]**pitation by Alcohol.** To 2 ml. of "egg albumin" solu- Picric acid is [illegible] of 95 per cent alcohol and mix. Does the precipitate

134. Millon's Test. Boil 2 ml. of "egg albumin" solution with a few drops of Millon's reagent. Compare your result with that obtained with tyrosine.

135. Hopkins and Cole Test. To 3 ml. of "egg albumin" solution add about 2 ml. of the Hopkins and Cole reagent (glyoxylic acid) and mix. Pour down the inclined test tube about 1 ml. of concentrated sulfuric acid. Observe the violet ring. This is due to the presence of tryptophan.

Glyoxylic acid (Benedict's modification) is made by adding to 10 g. of powdered magnesium, 200 ml. of distilled water, and, *gradually*, 250 ml. of saturated oxalic acid. Keep cooled by running water. When the reaction is over, filter, acidify with acetic acid and dilute to a volume of 1 liter.

Note: The color tests described above are not satisfactory for the detection of a[illegible] urine.

136. [illegible]rimble (phosp[illegible] Heat 5 ml. of "egg albumin" solution the cystine solution 1 ml. of [illegible]on and 3 drops of pyridine in boiling 10 per cent sodium sulfite, and, a[illegible] This is caused by free amino groups. reagent. The sulfite reduces the reaction proceeds best in neutral cysteine sulfonic acid (Clark, H. T.[illegible]n, or triketohydrindene hydrate is: The former substance, in the preser[illegible] the uric acid reagent. Cystine itself[illegible] structural formulas of cystine and of cy[illegible]

128. Unoxidized Sulfur. Boil some[illegible] concentrated sodium hydroxide and a few tion. Lead sulfide is formed.

129. Demonstration — Determination of Slyke Method (Van Slyke, D. D., *J. Biol. Chem.*[illegible]dd to 2 ml. samples of [illegible]cent mercuric chloride, 125 [1913]; **83**, 425 [1929]). This demonstration [illegible]te, ferric chloride, and up by the student and a diagram of the apparat[illegible]ipitate may redissolve the note book. For what purpose is amino nitrog[illegible]

Note: Primary amino groups give off gaseous [illegible]cipitate with "egg on by nitrous acid. Secondary and tertiary amino[illegible]OH. Why?

130. Glutathione. To about 5 ml. of ground [illegible]limin" precipitated suspension add 4–5 drops of a fresh solution of nitr[illegible]c acid, phospho- 2 g. of solid ammonium sulfate, and 3 ml. of 10 per [illegible]ric acid, and di- and shake. Now pour on an excess of ammonia.
color. This is a test for glutathione, the tripeptide [illegible]rosalicylic acid covered in yeast by F. G. Hopkins. Glutathione is glutam[illegible]ible to detect glycine and its structural formula is: [illegible]bright beam [illegible]nk.

Tungstic acid is a valuable precipitant used in blood analysis. One adds 1 volume of 10 per cent sodium tungstate, mixes with the protein solution, and adds, with constant rotation, 1 volume of two-thirds normal sulfuric acid. Try this with the "egg albumin."

139. Salting Out. To 2 ml. of "egg albumin" solution add 2 ml. of saturated ammonium sulfate solution. Does a precipitate form?

Add solid ammonium sulfate with shaking until the solution is saturated. Is the protein completely precipitated? Filter and apply the biuret test to the filtrate. Add concentrated sodium hydroxide in addition, as the alkali in the biuret reagent will be used up in reacting with ammonium sulfate to form ammonia. Animal globulins are precipitated by half-saturation with ammonium sulfate, animal albumins by complete saturation.

140. Heat Coagulation. Boil 3 ml. of "egg albumin" solution after mixing with 2 drops of concentrated sodium hydroxide. Repeat after mixing 3 ml. of "egg albumin" solution with 1 drop of 1 per cent acetic acid. Repeat, using this time 10 drops of 10 per cent acetic acid. Is there an optimum pH for the heat coagulation of "egg albumin"?

Heat 3 ml. of "egg albumin" with 1 drop of 1 per cent acetic acid and a pinch of sodium chloride. Salt causes the denatured albumin to precipitate out. A good reagent for the acidification of protein solutions preliminary to heat coagulation is composed of sodium acetate 10 per cent, and acetic acid 1 per cent. This is a "buffer" solution. It keeps the acidity constant. One milliliter of this buffer is sufficient for 5 ml. of urine.

141. The Distinction Between Denaturation and Coagulation. Heat a dilute solution of salt-free ovalbumin in boiling water for 5 minutes. Observe that the solution has become slightly opalescent, but has not precipitated. Now add some sodium chloride solution and note that the denatured albumin is precipitated. Heating salt-free albumin denatures it and changes it from a lyophilic to a lyophobic colloid. The particles of the latter are kept from uniting because they have electric charges. These charges are neutralized by the sodium ions. Salt-free ovalbumin can be prepared by dialyzing a solution of crystalline ovalbumin in a collodion membrane against distilled water for several days. The distilled water is changed each day and toluene is used as a preservative. If an electro-dialyzer is used, the salt can be removed within a few hours.

142. Precipitation by Alcohol. To 2 ml. of "egg albumin" solution add 5 ml. of 95 per cent alcohol and mix. Does the precipitate

redissolve upon adding a large excess (10 ml.) of water? Some proteins are very rapidly denatured by alcohol, others are not.

143. Heat 2 ml. of "egg albumin" solution with 2 drops of lead acetate solution and a few drops of concentrated sodium hydroxide. Compare this test with the result of experiment 128.

144. Introduction to Crystallization of Proteins. Crystallization of proteins has been of the greatest importance in changing the belief that proteins were ill-defined colloids of indefinite structure (the squash theory) to the view that they are definite chemical individuals with a regular structure which some day may be completely elucidated.

Although crystallization of a protein in itself is not evidence of purity, it is known at the present time that crystallization is the best method of purification. By repeated recrystallizations a protein usually can be rendered essentially pure, in the sense of being free from other proteins. Protein crystals, however, must contain large amounts of water, and if they have been prepared by salting out, large amounts of salt are present also. This salt of course can be removed by dissolving the crystals and dialyzing.

The fact that a protein will crystallize indicates some sort of regularity in the molecule. An enormous molecule such as that of a protein could not be expected to crystallize if it were in the form of a giant straight-chain polypeptide.

If crystals of a protein contain other proteins present as impurities, it is likely that a large part of the other proteins present are in solid solution with the protein in question. If one considers that it is possible for protein molecules to be built on similar patterns, but to have very slight differences in side groupings, it is theoretically possible to have a very large number of proteins together in solid solution in a crystal. Such crystals would appear homogeneous by many of the methods of testing known at the present time. We consider such a supposition rather improbable.

Proteins are delicate substances and will not stand the treatment ordinarily given to ordinary organic materials in crystallization. Heating to 50° C. often destroys them. Excess of solvents such as acetone, alcohol, dioxane, etc., may denature them. Excess of acid or alkali is to be avoided. The hands should be kept clean during the preparations, and the glassware must be absolutely free from dirt or cleaning solution. Sudden precipitation results in an amorphous precipitate, or at the best, submicroscopic crystals. Drying usually denatures a large part of the protein. If both salt and alcohol are present in high concentration,

denaturation is usually very rapid. If a protein solution or a suspension relatively free from salt is kept at room temperature for more than a day, putrefaction is very likely to result. Denaturation may also result from long standing at room temperature. If the crystals are kept even in the icebox in the presence of relatively large amounts of solvents such as acetone, alcohol, or dioxane, for long periods of time, they are likely to become surface-denatured and largely insoluble. The best way to keep most proteins is in solution in the icebox with a little toluene added for preservative, when this is known to be harmless to the protein. Some proteins are stable. For example, concanavalin A may be kept for long periods of time at room temperature in saturated salt solution.

145. Testing a Crystalline Preparation to Determine Whether it is Protein in Nature. The most rapid test is to add a fraction of a drop of crystal violet solution to a drop of the suspension of crystals and observe under the microscope. If the crystals are protein, they will take up the dye. Try this with the various protein crystals from the jack bean. If one employs highly-colored protein crystals, such as those of catalase or hemoglobin, it becomes difficult to observe whether or not the dye is taken up.

If enough of a preparation can be obtained, the unknown crystals are centrifuged off, dissolved in water containing salt or whatever buffer is necessary to dissolve them, and the usual tests for protein are run. Heat coagulation, and digestion by pepsin and other proteolytic enzymes are important.

146. Preparation of Crystalline Urease (Sumner, J. B., *J. Biol. Chem.* **69**, 435 [1926]).

Preparation of Jack Bean Meal (Kirk, J. S., and Sumner, J. B., *J. Ind. Eng. Chem.* **24**, 454 [1932]). Jack beans are coarsely ground in a large coffee mill and are put through the mill three times. The hulls, which contain no urease, are removed by allowing the material (after sifting out fine matter) to fall into a box through a blast from an electric fan. The air blast blows away the light hulls. The material is now ground in a mill constructed especially for this purpose. As the ground meal issues from the mill it is sifted in a box through No. 14 silk bolting cloth. The coarse material is then reground.

The students are asked to watch the whole process and to examine the product.

According to Nelson and Patterson of this laboratory the Mikro-Pulverizer (Type SH, Pulverizing Machinery Company, Summit, N. J.)

can be used to prepare jack bean meal. The machine is set to operate with a maximum amount of air being drawn through during the grinding. The beans are ground directly using the finest mesh screen. The resulting meal is as good or better than that prepared as described above.

Fill 500 ml. graduated cylinders to the 160 ml. mark with purified acetone.* Dilute to 500 ml. with glass-distilled water and stir well to mix, using a glass rod. Bring the temperature to 24–28° C. Weigh 100 g. portions of jack bean meal into 1000 ml. beakers. Now pour 500 ml. of the diluted acetone into each beaker and at once stir with a clean wooden stick or paddle until the meal is evenly suspended in the acetone. Pour the material on a 32 cm. filter (Schleicher and Schüll, No. 595, or Whatman, No. 1) and allow to filter at room temperature into 500 ml. graduated cylinders until 100 to 150 ml. have filtered through. This time interval allows enough acetone to evaporate so that only a slight amount of amorphous impurity is precipitated in the filtrate later with the urease crystals. Now place the filtering material in an ice chest at 3–6° C. and allow to continue filtering.

After standing at this temperature for 20–24 hours, the urease crystals in the filtrate are centrifuged off, using centrifuge tubes of 50, 250, or 500 ml. capacity. Much time will be saved if a centrifuge employing tubes of 500 ml. capacity is available. Using 50 ml. tubes, it is usually necessary to centrifuge at 2000–2500 revolutions per minute for 10–15 minutes; with 500 ml. tubes it is necessary to centrifuge 30–40 minutes. After centrifuging, the supernatant liquid is carefully poured off and the tube, containing the urease crystals sticking to the bottom, is filled with more cold filtrate containing urease crystals and centrifuged again. We have considered it necessary to cover the centrifuge tubes with paper caps, or flanged cork stoppers, in order to prevent dirt from entering the tubes during centrifuging as well as to prevent evaporation of acetone.

After all of the filtrate has been centrifuged, the supernatant liquid is poured out and the tubes are allowed to drain for a few minutes, inverted on several clean filter papers. The urease crystals are then dissolved by stirring with redistilled water. The amount of water employed is usually 3 ml. for each 100 g. of meal employed. It is advisable to follow solution of the crystals by observing a *small* drop of the material under the microscope.

* If technical acetone is used, this should be first redistilled from a mixture of fused calcium chloride and soda lime.

147. Recrystallization of Urease (Dounce, A. L., *J. Biol. Chem.* **140**, 307 [1941]). Either centrifuge, or filter the urease solution in the ice chest through a Whatman No. 5 filter paper until the filtrate is clear. To every 20 ml. of the filtrate add 1 ml. of 0.5 *M* citrate buffer, pH 6.0. Next add 0.2 volume of pure acetone with constant stirring. The solution is now placed in the ice box. Crystallization will be nearly complete after one-half hour. The crop of crystals can be increased somewhat by adding acetone, a few drops at a time, until the solution is about 25 per cent acetone.

The 0.5 *M* citrate buffer is prepared by adding 95 volumes of 0.5 *M* trisodium citrate to 5 volumes of 0.5 *M* citric acid.

148. Concanavalin A (Sumner, J. B., and Howell, S. F,. *J. Bact.* **32**, 227 [1936]). After one has prepared crystalline urease (Exp. 146) one can employ the residue on the filter paper for the preparation of three other crystallizable jack bean proteins, namely concanavalin A, concanavalin B (Exp. 149) and canavalin (Exp. 150).

To prepare concanavalin A, extract every residue from 100 g. of meal with 500 ml. of 30 per cent alcohol and filter at room temperature.* This preliminary treatment removes substances which interfere with later procedures. After the liquid has all drained off, discard the filtrate and extract the residue with 400 ml. of solution which contains 4 g. of NaCl, 1.5 g. of KH_2PO_4 and 4 g. of Na_2HPO_4. Filter on a fluted filter at room temperature. Do not add any toluene, since this may cause the concanavalin A to crystallize out before it has filtered through. Now re-extract the residue on the filter with 250 ml. of 5 per cent NaCl. Unite the two filtrates, add toluene, and dialyze either in viscose tubes † or 4 or 5 collodion sacks in the ice chest for 48 hours, changing the outside water several times.

The dialysis should cause most of the concanavalin A to crystallize out, leaving the other crystallizable globulins in solution. However, if the dialysis is continued too long some concanavalin B crystallizes out as needles and some canavalin separates as spheroids. Remove the material and place it in a beaker. Examine a *small* drop of the suspended material under a microscope to see what has precipitated. Add a small drop of 5 per cent NaCl and note whether any material dissolves. Anything that does dissolve is canavalin.

Centrifuge off the concanavalin A, decant and save the super-

* Cover the funnel to prevent undue evaporation.

† Cellophane tubing may be used.

natant liquid. Wash the concanavalin A once with 10 per cent NaCl. Centrifuge down (save the washing if you have decided that an appreciable amount of canavalin is present) the concanavalin A and dissolve it in the least possible amount of saturated NaCl, that also contains some neutral phosphate buffer, by warming to 40–50° C. It will be necessary, probably, to add some solid NaCl to saturate the solution fully. Filter and dialyze to recrystallize. To avoid undue dilution allow the sack with contents to just barely touch the water used in the dialysis.

Preparation of 9.6 *Per Cent Neutral Phosphate Buffer.* Weigh out 28 g. (0.206 moles) of anhydrous KH_2PO_4, and 68 g. (0.478 moles) of anhydrous Na_2HPO_4, and place in a liter Erlenmeyer flask. Add 900 ml. of redistilled water and then heat to boiling. Pass in H_2S *in the hood* until saturated (an hour or so). Filter into about 200 ml. of boiling redistilled water, boil *in the hood* until free from H_2S, cool, and dilute to one liter.

In working with proteins, particularly certain enzymes, great care must be taken to avoid heavy metals. The use of redistilled water, redistilled acetone, and specially purified reagents is not always necessary to secure crystals of a protein, but is absolutely necessary for quantitative work with sensitive enzymes such as urease.

149. Concanavalin B (Sumner, J. B., *J. Biol. Chem.* **37**, 137 [1919]). To the filtrate after removal of the once-crystallized concanavalin A, add enough *N* acetic acid to bring the pH to about 5.1. (An indicator can be used to determine this point roughly.*) Concanavalin B and canavalin are precipitated. After 2 hours standing in the ice chest filter off this precipitate, place in a beaker, and add enough 0.1 *N* NaOH, with stirring, to give a neutral reaction. (Use an indicator.†) To insure neutrality, a small amount of neutral phosphate buffer may be added also. This procedure dissolves the canavalin and leaves the concanavalin B as precipitate. The concanavalin B is filtered off and is dissolved in 10 per cent NaCl. Recrystallization is brought about by dialysis.

150. Canavalin (Sumner, J. B., and Howell, S. F., *J. Biol. Chem.* **113**, 607 [1936]). The dissolved canavalin is precipitated as spheroids by adding enough *N* acetic acid to give a pH of 5.1 (indicator); is filtered off and dissolved by adding 0.1 *N* NaOH as before. The solution is analyzed by drying and weighing an aliquot and is then diluted to give

*E.g., yellow-orange to chlor phenol red, but blue to brom cresol green.

†E.g., blue-green to brom thymol blue.

6 per cent. To crystallize, adjust the pH to 6.5 with dilute acetic acid (indicator, or better, glass electrode), and add to one volume of this canavalin solution one volume of one per cent Fairchild's trypsin and a little toluene. Stopper and place the solution in an oven at 37° C. and allow to stand over night.

The crystals of canavalin that will form by this procedure are soluble in distilled water at pH 6.5, but are insoluble in 0.2 to 1.0 per cent NaCl. They are soluble in NaCl more concentrated than 1 per cent. To recrystallize, centrifuge off the crystals, suspend in distilled water, dialyze until dissolved, and add a small amount of dilute NaCl or $CaCl_2$ solution. The safest way, however, is to dialyze a small amount of the canavalin solution against 0.5 per cent NaCl.

151. Preparation of Crystalline Oxyhemoglobin. Oxyhemoglobin was one of the first proteins to be crystallized. It is one of the most important examples of conjugated proteins. The oxyhemoglobin of a large number of animal species has been crystallized.

Centrifuge 100 ml. of oxalated, or defibrinated, cow blood (horse blood is still better) and remove the supernatant liquid by suction through a bent tube leading to a suction flask interposed between the tube and the suction pump. *Do not allow any liquid to pass into the suction pump as it is likely that the pump will be stopped up and thus made useless.* Stir the centrifuged corpuscles with an equal volume of 0.9 per cent NaCl containing a trace of neutral phosphate buffer, and centrifuge again. Repeat twice more, removing the supernatant each time. Now, in order to hemolyze, add about 0.3 g. of saponin. Stir well and dilute with one volume of distilled water. Filter through a fluted filter, changing this several times. Do this in the ice chest.

Now, while stirring rapidly, add to the cold oxyhemoglobin filtrate small quantities (about 10 ml.) of ice cold 95 per cent ethyl alcohol, waiting for one-half to one hour after every addition of alcohol. Do not add too much alcohol, or the oxyhemoglobin will be denatured. Examine the crystals under the microscope. Do not allow the preparation of crystallized oxyhemoglobin to warm room temperature.

Filter off the crystals by suction, using a chilled Büchner funnel. Dissolve the crystals by stirring with 70 ml. of distilled water and filter by gravity to free the solution from denatured impurities. Recrystallize as above by chilling and adding ice-cold alcohol.

152. Crystallization of Ferritin (Laufberger, V., *Bull. soc. chem. biol.* **19**, 1575–1582 [1937]). Ferritin is a very remarkable protein obtained from horse spleen. It has an iron content of 20 per cent.

It is also present in the liver of the horse. Such an iron content would require one atom of iron for every two or three amino acid residues. The protein is easy to prepare. It may possibly serve as a place of storage for iron.

Grind horse spleen ten times in the electric meat grinder. About 1 kg. of spleen will give a good yield of ferritin. For every kilogram of ground spleen, add 1500 ml. of distilled water. Squeeze out most of the extract through three layers of cheesecloth. Place the residue, with the cheesecloth, in canvas and press out the rest of the juice in the Buchner press. Combine the two extracts and heat to just 80° in a water bath and then at once refilter. (*Note:* As a rule such heating will at least partly denature a protein. In general it is to be avoided.) Add 30 g. of solid ammonium sulfate slowly with stirring to every 100 ml. of filtrate. Chill in the ice chest for one-half hour and then filter off the precipitate. Dissolve the precipitate in distilled water to give a moderately dilute solution. Then add 10 g. of solid ammonium sulfate to every 100 ml. of this solution and chill in the ice box for half an hour or longer.

Centrifuge the precipitate in large centrifuge tubes. Then to the centrifuged solution add as much ammonium sulfate as had just been added to precipitate impurities. Chill in the ice box for at least an hour, and then centrifuge down the crude ferritin. Discard the supernatant. Dissolve this crude ferritin in the least possible amount of water and then add twice as much water as there is saturated solution of ferritin. Filter. Add to the filtered solution one-half its volume of a 20 per cent solution of cadmium sulfate (3 $CdSO_4.8\ H_2O$). Place the material in the ice chest. Crystallization begins almost immediately. The crystals grow to form beautiful orange-brown octahedra. There is practically no amorphous impurity present.

To recrystallize, centrifuge down the crystals and dissolve in redistilled water. Solution is quite slow. Filter and recrystallize as above with cadmium sulfate.

153. Crystallization of Egg Albumin (Hawk and Bergeim, Eleventh Edition, page 165; Method of Cole, *Proc. Soc. Exptl. Biol. Med.* **30**, 1162 [1933]). Egg albumin was among the first proteins to be crystallized. A great amount of work has been done on it, mostly of a physicochemical nature. To render it approximately pure, at least three recrystallizations are required.

Fresh eggs are broken and the whites are carefully separated from the yolks. The whites are then stirred to break up the membranes and are filtered through cheesecloth. To each nine volumes of egg white,

one volume of 1 *N* acetic acid is added slowly with constant stirring. This should bring the pH to about 5.0 or less. A small amount of precipitate which has formed is removed by straining the material again through cheesecloth. This strained solution may then be filtered through a fluted Schleicher and Schüll, No. 595 filter paper if it is not clear.

Enough saturated ammonium sulfate is now added to this clear solution to bring the material to 40 per cent saturation with ammonium sulfate. The slight precipitate formed is filtered off. Saturated ammonium sulfate is added to the clear filtered solution until there is considerable opalescence. The solution is then allowed to stand with occasional stirring at room temperature or in the ice chest until crystallization is complete (about 5 days are required).

The crystals are filtered or centrifuged and are washed once with ammonium sulfate of a concentration which just fails to give a turbidity with the mother liquor. They are then dissolved in distilled water (enough to dissolve them completely) and the solution is filtered clear. To induce crystallization one adds a buffered saturated solution of ammonium sulfate until a rather high opalescence is produced. This buffered ammonium sulfate solution is prepared by mixing one volume of acetate buffer at pH 4.8 (*N* acetic acid plus *N* sodium acetate) with nine volumes of saturated ammonium sulfate solution, and then resaturating the solution by adding solid ammonium sulfate. Further recrystallizations are performed in the same way.

This method can be used to get crystalline serum albumin from fresh horse serum. Serum albumin, however, apparently must be recrystallized a great many times in order to get it pure (Hewitt, L. F., *Biochem. J.* **30**, 2229 [1936]).

154. Preparation of Crystalline Beef Liver Catalase (Sumner, J. B., and Dounce, A. L., *J. Biol. Chem.* **121**, 417 [1937]). Catalase was the first iron-containing enzyme to be crystallized. It is most readily obtained from beef liver. Crystalline beef liver catalase contains two hematin residues per molecule and has a *Kat. f.* of about 30,000. It also contains two other prosthetic groups apparently related to hematin, which produce a blue color upon addition of acetone and HCl.

Put very fresh beef liver through the electric meat grinder ten times and mix 300 g. portions with 400 ml. portions of 35 per cent dioxane (140 ml. of dioxane diluted to 400 ml. with distilled water). Five pounds of liver give a good yield of catalase. The dioxane is obtained by freezing Eastman technical dioxane and allowing it partly to melt. After about one-tenth of the volume has melted, this is poured off and

discarded, and the remaining solid is melted and used.

After extracting for about one hour at room temperature, filter the liver suspension through Schleicher and Schüll, No. 595 fluted filters into 500 ml. cylinders, in the cold room or in the ice box. The next day, add to every 100 ml. of filtrate 20 ml. of dioxane and allow to stand in the ice box for twelve hours. Filter through Schleicher and Schüll, No. 595 fluted filters and to every 100 ml. of filtrate add 10.2 ml. of dioxane. Stir. Allow the material to stand for another twelve hours. The precipitated crude catalase is now filtered off on the same kind of fluted filters used before, and is allowed to drain fairly dry, *i.e.*, practically all of the liquid is allowed to drain off, but the precipitate must *not be allowed to dry out.* The precipitate is scraped off with a steel spatula and placed in a beaker. This is then well mixed with enough water to form a thin cream and filtered. The residue on the filter still contains a large amount of catalase. It is mixed with more water and again filtered. The residue is now mixed with dilute neutral phosphate buffer and filtered again. This extraction with phosphate buffer and filtration is repeated once more.

The combined filtrates may be opalescent with glycogen. This is a good sign, since it shows that the liver used was fresh. A little saliva is added (1–2 ml.) to digest this glycogen. The dark greenish-brown solution is now dialyzed in the ice chest until a whitish precipitate forms. This is centrifuged off and discarded. The supernatant is dialyzed longer until crystals of calalase form. These are centrifuged off.

If old liver is used for the preparation of catalase much of the catalase is likely to be precipitated when one adds 20 ml. of dioxane to every 100 ml. of the first filtrate. This catalase can be recovered by scraping the precipitate from the filter paper and extracting several times with water. Beginners should always investigate to see whether the catalase has come down in this precipitate. This can be done by testing a minute quantity of the precipitate with 3 per cent hydrogen peroxide.

If one has a catalase solution which apparently is too dilute to crystallize out upon dialysis one can obtain crystalline catalase from this solution by adding cautiously saturated ammonium sulfate until a faint permanent turbidity is produced. The solution is then placed in the ice chest to crystallize. More ammonium sulfate is added from time to time.

Catalase can be recrystallized by dissolving the crystals in a small volume of 0.5 *M* phosphate buffer pH 7.4 and enough sodium chloride to make a 10 per cent solution. After the catalase dissolves it is dialyzed.

155. Separation of the Prosthetic Groups of Catalase. Pipette slowly and with vigorous stirring one ml. of a concentrated suspension of dialyzed beef liver catalase into 25 ml. of acetone containing 3 ml. of *N* HCl. Filter off the acid-denatured protein. Observe the blue color of the solution. This blue-colored substance is not present in the catalase molecule, but has some precursor, probably similar to hematin. Allow the solution to evaporate somewhat, and then observe the hemin with the pocket spectroscope. Evaporation of nearly all of the acetone will cause the hemin to crystallize. Observe the crystals under the microscope and draw them. The blue color remains in the supernatant solution after centrifuging down the hemin crystals, but it is not stable and fades slowly.

156. Preparation of Crystalline Tobacco Mosaic Disease Virus (Stanley, W. M., Ergebnisse der Physiologie, biologischen Chemie und experimentellen Pharmakologie **39**, 294 [1937]). The tobacco mosaic disease virus was the first virus to be crystallized. If the virus is really identical with the crystalline protein, as seems almost certain, its crystallization (first reported by Stanley, *Science* **81**, 644 [1935]) is one of the major accomplishments of modern biochemistry.

Infected tobacco plants are cut and frozen. The frozen plants are put through the meat grinder until a pulp is formed, and the juice is pressed out by a press. The pulp is extracted at pH 7.0 with 0.1 *M* phosphate buffer. This extract is added to the original press juice. The pH is brought to 6.7–7.0 by adding 0.1 *N* NaOH. Now filter through Schleicher and Schüll, No. 595 filter paper, or other similar filter paper. This will take about 24 hrs. Now filter through a layer of celite about one-half inch thick on a Büchner funnel. The celite filter cake is scraped with the flattened end of a spatula from time to time in order to speed the filtration. The virus fraction is separated from the filtrate by addition of 30 per cent by weight of solid ammonium sulfate, and is removed by filtration through filter paper (Schleicher and Schüll, No. 595 fluted), or by centrifuging in large tubes, or by filtering through a thin layer of celite on a Büchner funnel. The precipitate is dissolved in sufficient water to give about a one per cent solution of protein, is adjusted to pH 7, and is again precipitated with solid ammonium sulfate, this time about 11 per cent by weight. If celite has been used in this filtration, it must be removed by filtration before the precipitation. The precipitation by ammonium sulfate just described is repeated two or three times until the protein precipitate is only slightly colored. The precipitate is then dissolved in water and the pH of the solution is adjusted to 4.5 with

10 per cent acetic acid, using an indicator. This causes precipitation of the protein, which is filtered off on a thin layer of celite, or is centrifuged. If celite has been used, it is removed by filtration on an ordinary Büchner funnel, after the filter cake has been suspended in water and adjusted to pH 7. If the centrifuge tubes were used, the material is simply dissolved in water, and the pH adjusted to 7.0, followed by ordinary gravity filtration, or recentrifugation. The filtrate will be opalescent, practically colorless, and will contain about 80 per cent of the virus of the starting material. Crystallization is induced by adding sufficient saturated ammonium sulfate solution to cause a slight cloudiness, then sufficient 10 per cent solution of acetic acid to bring the pH to about 5.5. Finally more ammonium sulfate solution is added dropwise over a period of several hours until the protein is out of solution. The crystals are very small.

The crystalline protein is centrifuged down, dissolved in water at pH 7.0, and recrystallized, using the method just described.

157. Crystallization of Edestin. Edestin is probably one of the easiest proteins to crystallize, provided that defatted hemp seed is available. However, the hemp seed now available in this country has been sterilized to prevent its use for the production of marijuana. This sterilized seed gives low yields of edestin.

Hemp is ground in a coffee mill and is reground to break up all of the grains. The fat is then extracted by mixing with petroleum ether (danger of fire) and pouring on a filter. The material is washed 3 or 4 times with petroleum ether. The meal residue is then dried by spreading it out on a large filter paper exposed to air.

Extract 20 g. of the defatted residue with 100 ml. of 5 per cent sodium chloride at 60° C. Now filter through filter paper *using a hot water funnel at* 60° *C.*, so that the temperature will not drop and allow the extracted edestin to separate out. Collect the filtrate in a 250 ml. Erlenmeyer flask. Finally heat the filtrate to 60° C. in a pail of water and rotate the flask until the edestin has dissolved. Do not allow the temperature to rise above this point. Leave the flask in the bath to cool very slowly, as the size of the crystals will depend on the rate of cooling. Next day observe under the microscope. Is the edestin soluble in water? In cold 5 per cent sodium chloride?

158. Gliadin. Make a very stiff dough by adding a little water to 25 g. of wheat flour in a large porcelain evaporating dish. Knead thoroughly and let stand for at least one-half hour, then wash out the starch by kneading under water. Continue until nearly all of the starch has been washed out and a rubbery mass of gluten remains.

Cut up the gluten with scissors, allowing it to fall into a Erlenmeyer flask containing 100 ml. of 80 per cent ethyl alcohol. Mix occasionally and let stand overnight. The alcohol will be diluted to about 70 per cent by the water in the gluten. This strength of alcohol dissolves the prolamine "gliadin." Pour off the extract. Cut the gluten up finer and re-extract with 100 ml. of 70 per cent alcohol. Add the second extract to the first and evaporate to a syrupy consistency on the steam bath in a porcelain evaporating dish. With this material perform the following tests:

1. Biuret.
2. Solubility in water and in salt solution.
3. Solubility in 2 per cent sodium hydroxide.
4. Solubility in 0.4 per cent hydrochloric acid.

A sample of spring wheat was found by Osborne to contain:

Glutenin	4.68 per cent
Gliadin	3.96 " "
Globulin	0.62 " "
Albumin	0.39 " "
Proteose	0.21 " "

159. Glutenin. To the residue left in experiment 158 after extraction with 70 per cent alcohol, add 50 ml. of 0.2 per cent NaOH and stir until most of the glutenin has dissolved. Filter through a wad of cotton. Neutralize some of the filtrate with tenth normal hydrochloric acid and note that at a certain pH the denatured glutenin reprecipitates and that the precipitate is soluble in an excess of acid.

Both gliadin and glutenin are insoluble in water but possess the ability of imbibing water and forming an elastic mass (dough).

160. Keratin. Test the solubility of keratin (wool) in weak alkali, weak acid, strong alkali, and strong acid. Try the unoxidized sulfur test.

161. Gelatin. Is gelatin soluble in cold water? Cold dilute acid? Cold dilute alkali? Hot water? Does it gel upon cooling? Does gelatin give Millon's test? The Hopkins and Cole test? The unoxidized sulfur test? What amino acids are lacking in gelatin?

Note: Do not boil a concentrated gelatin solution over a free flame or it will crack the beaker. Do not let gelatin dry in a beaker or it will crack the bottom of the beaker.

162. Casein. Is casein soluble in water? In dilute acid? In dilute alkali? Is it precipitated upon neutralization?

163. Make a solution of sodium caseinate by adding a slight excess of 0.1 *N* sodium hydroxide to a little casein and mixing. Now,

cautiously add, drop by drop, 0.1 *N* hydrochloric acid with rapid stirring until the solution is just red to methyl red. Filter through cotton. Is the casein precipitated from this solution by adding 1 ml. of 5 per cent calcium chloride solution? Could this be used as a test for casein? Is it precipitated by adding 3 drops of trypsin solution and warming?

164. Proteoses and Peptones. To 5 ml. of 5 per cent proteose-peptone solution (Witte's) add solid ammonium sulfate, shaking and warming to 20° C. until saturated. Filter. Is the filtrate foamy? To 1 ml. of the filtrate add 5 ml. of water and an excess of picric acid. If there is no precipitate proteoses are absent, having all been precipitated by the ammonium sulfate. To 1 ml. of the filtrate, add 5 ml. of water and some fresh 10 per cent tannic acid solution. Add only a few drops of tannic acid solution at a time. An excess of the tannic acid solution will dissolve the precipitate. Tannic acid precipitates peptones, while picric acid does not.

Dissolve the precipitate of proteoses, produced by salting out with the ammonium sulfate, in water, and try the biuret test on a part of it. To another part add saturated aqueous picric acid solution. Does the precipitate dissolve on heating and reappear on cooling? This is a good test for proteoses. It is also given by gelatin.

Note: Witte's peptone may not be available and certain American preparations are nearly free from proteoses. A satisfactory proteose-peptone may be prepared from fibrin. 30 g. of fibrin is soaked in 1000 ml. of water. Then 8 g. of concentrated HCl and 2 g. of 1 : 3000 pepsin are added. Mix, and after an hour or so, make alkaline to phenolphthalein with NaOH. Allow to stand for a time to inactivate the pepsin. Adjust to a green color to brom cresol green by adding 1 *N* HCl and filter. The filtrate may be used directly, or a dry preparation may be obtained by adding two volumes of 95 per cent ethyl alcohol. Filter off the precipitate, wash it with 95 per cent alcohol and allow it to dry. Still more proteose-peptone may be obtained by evaporating the filtrate from the alcoholic precipitation to dryness on a steam bath. This product will turn dark, but is satisfactory for qualitative tests. Proteose-peptone prepared according to these directions contains only small amounts of peptone. It is advisable to add more peptone to the preparations before using them.

165. Are proteoses coagulated by boiling?

166. Protein Unknowns. Apply for two protein unknowns by handing in two clean, dry test tubes with a label bearing a number

and your name. Each unknown may contain any one or more of the following:

Albumin	Keratin
Globulin	Casein
Gelatin	Proteose-Peptone

The student must bear in mind the fact that most solid specimens of albumin and globulin are not wholly soluble in water or salt solution because of having been partly denatured during the process of drying.

Scheme for the Analysis of Protein Unknowns (Solutions).

Add a few drops of the solution to 10 ml. of water. A precipitate indicates globulin. If there is no precipitate, add a drop of 2 per cent acetic acid to the diluted solution. Mix. If there is a precipitate, casein or globulin is indicated. Add about 100 mg. of solid sodium chloride to one-half of the turbid suspension. If the suspended material dissolves globulin is present. If the material dissolves readily, add 1 ml. of acetate buffer, pH 4.5, and heat in boiling water for 5 minutes. A coagulum indicates globulin or albumin. Filter. The filtrate may contain proteose-peptone. Add excess picric acid to the filtrate and cool. A precipitate indicates proteose-peptone, provided gelatin was absent.

If casein was indicated as being present, test for casein by making the original solution neutral to phenol red, if not already neutral, and adding to it some calcium chloride solution. Note whether a heavy precipitate forms.

Note: If peptone is present, this test cannot be employed since the peptone may prevent the precipitation of the casein by the calcium.

Make another part of the solution just acid to methyl red, filter, and add a trace of solid trypsin. Mix, and warm gently. A precipitate indicates casein.

Albumin can be detected by adding 2 ml. of acetate buffer of pH 4.8 to 2 ml. of the original unknown and dialyzing against distilled water for 2 hours. The globulin and casein will precipitate. Now filter, and heat the filtrate in boiling water. A precipitate indicates albumin.

Proteose-peptone can be detected by dialyzing a solution of about 1 g. of the unknown in 5 ml. of water in a cellophane membrane against about 5 ml. of water. After one or two hours, remove the membrane and test a part of the outer liquid with the biuret reagent and another part with picric acid.

As a confirmatory test for globulin, it may be well to dialyze some of the solution against $N/1000$ acetic acid (or better still, against $N/1000$ acetate buffer of pH 4.8). If a precipitate is formed which is readily soluble in 1 to 5 per cent NaCl, the precipitate is globulin.

Scheme for Analysis of Solid Unknown Proteins

To 0.5 g. of salt-free [1] unknown, add 5 ml. of distilled water. Rub up with a stirring rod and decant through filter paper.

Filtrate
May contain:
Albumin
Proteose-peptone
Gelatin (traces)

Residue
See following page for analysis.

Add 2 ml. of pH 4.8 acetate buffer and heat.

Filtrate
May contain:
Proteose-peptone
Gelatin (traces)

Precipitate
If present, indicates albumin. *Discard.*

Add picric acid solution.

A precipitate which redissolves on heating indicates proteose-peptone or gelatin.

[1] *Note:* The solid unknowns will be salt-free. If salt were present, the first filtrate would have to be dialyzed until all globulin had precipitated, before testing for albumin.

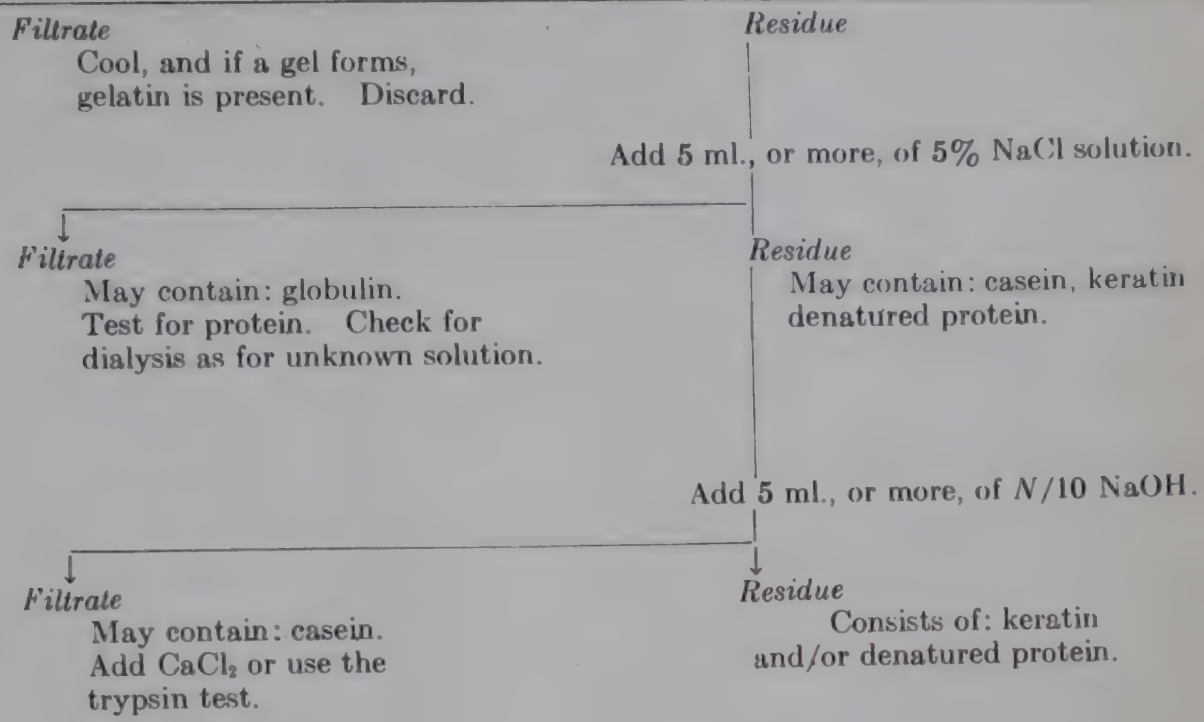
As a confirmatory test for proteose-peptone, dialyze a strong solution, or suspension, of the original unknown against 5 ml. of distilled water. Test the water *outside* the cellophane sac for proteose-peptone after 1 or 2 hours, or longer. This method avoids interference by gelatin. It is also useful for the liquid unknown.

Analysis of Solid Unknown Proteins, cont'd.

Residue
May contain: gelatin, globulin, casein, keratin, denatured protein.

Add 5 ml. of water at 50° C. and filter.

(continued on following page)



OXYHEMOGLOBIN, HEMIN, AND RELATED COMPOUNDS

167. Crystalline Rat Oxyhemoglobin. Place a small drop of oxalated rat blood on a glass slide, add a drop of 5 per cent saponin, mix and warm gently until the erythrocytes have been hemolyzed, as can be seen by the transparent appearance of the blood. Now place a cover glass over the preparation and wait 20 to 30 minutes. Examine under the microscope. One will see crystals of oxyhemoglobin.

Rat oxyhemoglobin is rather insoluble in water and crystallizes soon after it escapes from the erythrocytes. Apparently the stromata keep it from crystallizing within the erythrocytes.

168. Oxyhemoglobin. Place one drop of oxalated or defibrinated blood (from any species of animal) in a test tube. Add 3 ml. of water Mix to lake and observe the absorption bands of oxyhemoglobin, using a pocket spectroscope. Now dilute the blood to about 6 ml. of volume and again observe. Concentrated oxyhemoglobin shows only one band while dilute oxyhemoglobin shows two bands.

169. Hemoglobin. Reduce the oxyhemoglobin in the test tube by adding a few mg. of sodium hydrosulfite, $Na_2S_2O_4$ (dithionite), and mixing. Now observe the single absorption band of hemoglobin. Notice that the color of the hemoglobin as seen by the unaided eye is purplish.

170. Carboxyhemoglobin. Place some dilute oxyhemoglobin in a test tube and some dilute carboxyhemoglobin (furnished by the instructor) in another test tube. Observe that the carboxyhemoglobin is cherry red in color. Now add about 1 ml. of 0.1 *N* sodium hydroxide to each tube and also a very few grains of sodium hydrosulfite. Mix and observe that while the oxyhemoglobin is reduced to hemoglobin, the carboxyhemoglobin is not reduced.

171. Denatured Globin Hemochromogen. Place a drop of defibrinated, or oxalated, blood in a test tube. Add 3 ml. of water and 1 ml. of 0.1 *N* hydrochloric acid. Note that the color changes to brown. The hemoglobin has been denatured by the acid. The solution contains acid hematin which has a band in the red part of the spectrum. Now add 1 ml. of 0.1 *N* sodium hydroxide. The denatured hemoglobin will precipitate. Now add about 2 drops of 10 per cent sodium hydroxide. The solution now contains alkaline hematin. The absorption bands can be seen with some difficulty with the pocket spectroscope. Now add about 10 mg. of sodium hydrosulfite. Note that the color changes from brown to pink. The compound present is the denatured globin hemochromogen. Observe the absorption bands with the spectroscope. The alpha band (the one nearest the red end) is more intense than the beta band (the second one).

172. Pyridine Hemochromogen. In the previous experiment the reduced hematin in alkaline solution united with denatured globin to form denatured globin hemochromogen. Reduced hematin in alkaline solution will unite readily with various proteins, pyridine, nicotine, certain amino acids, etc.

Prepare pyridine hemochromogen by placing 2 ml. of a solution containing 0.5 mg. of hematin per ml. in a test tube and adding 2 drops of pyridine, about 1 ml. of 10 per cent sodium hydroxide, and a few mg. of sodium hydrosulfite. Observe the absorption bands.

Using this test it is easy to detect as little as 0.01 mg. of hemin spectroscopically. The hematin solution is made by dissolving a small amount of pure hemin in 0.1 *N* sodium hydroxide. This solution is not stable.

173. Methemoglobin. Place 1 drop of oxalated, or defibrinated blood in a test tube. Add 3 ml. of water and one drop of potassium ferricyanide solution. Note that the color of the hemoglobin changes to brown. The material is methemoglobin. Observe the absorption band. Now reduce the methemoglobin to hemoglobin by adding the least possible quantity of sodium hydrosulfite. Add also 2 ml. of 0.1 *N* sodium hydroxide to prevent the solution from becoming acid

later when the hydrosulfite is oxidized. Now shake the hemoglobin solution with air to oxidize it to oxyhemoglobin.

174. Hemin Crystals—Teichmann's Test for Blood. Place one drop of blood on a glass slide and add a very small crystal of sodium chloride. Evaporate very carefully to dryness over a flame, but do not boil material. Add a drop of glacial acetic acid and cover with a cover glass. Warm carefully over a flame until the acetic acid is just brought to boiling. Allow to cool and observe the hemin crystals under the microscope. If no crystals are visible, run more glacial acetic acid under the cover glass and heat again.

175. Hematoporphyrin. Observe the color of a solution of hematoporphyrin in dilute hydrochloric acid prepared by the instructor and note the absorption bands in the large spectroscope. If it is possible, observe the effect of ultra-violet light on a dilute hematoporphyrin solution in a darkened room. Rub some hematoporphyrin powder on a finger and observe under a source of ultra-violet light.

176. Preparation of Hematoporphyrin. Place about 0.5 g. of dry, well-powdered hemin or hematin in a 20–30 ml. glass bottle with a constricted neck. In the hood, pour in about 10 ml. of glacial acetic acid saturated with dry hydrobromic acid. Seal the bottle with a blast lamp, allow to cool and then mix well. Allow to stand for 7 to 10 days. Now open the seal and (in the hood) pour and wash out the contents, diluting with 150 ml. of distilled water. Filter and add cautiously to the red filtrate just enough ammonia to form a voluminous chocolate precipitate of hematoporphyrin (pH 0.6). Filter this off and wash repeatedly with distilled water until the washings are colorless. Now, remove the material to a clean watch glass and dry in a desiccator or in an oven at a low heat. Powder and place in a small bottle. When hematoporphyrin solution is to be prepared dissolve some of the powder in dilute hydrochloric acid.

The product may be purified and obtained as the crystalline dihydrochloride by means of a method given by Fischer and Orth (Fischer, H., and Orth, H., Die Chemie des Pyrrols, Vol. II, Band I, Leipzig [1937]).

Preparation of Glacial Acetic Acid Saturated with Hydrobromic Acid. Place 250 ml. of 85 per cent ortho-phosphoric acid in a 7-inch evaporating dish and heat over a burner in the hood for 2 hours to drive off the water. Cool and pour into a 1 l. pyrex flask. Cautiously add 5 to 10 g. of phosphorus pentoxide to remove the last traces of water. Next, add 120 g. of well-dried sodium bromide and fit the flask with a cork stopper and a glass tube with two 90 degree bends.

The tube should be about 15 mm. in diameter and the outside descending arm should be 20 inches long. Heat in the hood very gently to evolve hydrobromic acid gas and pass this gas into 50 ml. of glacial acetic acid in a glass bottle cooled by ice. The end of the delivery tube should just touch the surface of the acetic acid. Continue the heating for about 2 hours, or until the density of the solution is 1.41. Now stopper the bottle with a well-fitting glass stopper.

177. Determination of Position of Absorption Bands of Hematin Compounds. With the assistance of the instructor, employ the large spectroscope to determine the centers of the absorption bands for oxyhemoglobin, reduced hemoglobin, carboxyhemoglobin, pyridine hemochromogen, etc. First adjust the spectroscope so that the scale reads accurately in wave lengths of light. Do this by using a sodium flame and gently tapping the glass prism with a pencil until the two sodium lines are just on the cross hairs when the scale on the drum is set at 5894.5 Å.

Location of Absorption Bands of Hematin Compounds

	Location of Bands mμ	*Character of Bands*
Hemoglobin	559	Distinct
Oxyhemoglobin	579 and 542	Very distinct
Carboxyhemoglobin	570 and 542	Very distinct
Methemoglobin (acid)	633	Fairly distinct
Methemoglobin (alkaline)	597, 574, 536	Indistinct
Hematin (acid)	642	Fairly distinct
Hematin (neutral or alkaline)	612, 534	Indistinct
Pyridine hemochromogen	555	Alpha band, extremely distinct
	523	Beta band, fairly distinct
Hematoporphyrin (acid)	592 and 550	Fairly distinct
Hemin in acetone	641	Distinct
	543	Fairly distinct
	510	Hardly visible with ordinary electric light.
Crystalline catalase	628, 538	Difficult to see unless the solution is concentrated.
	502	Very indistinct

The results can be expressed as wave lengths of light. These wave lengths are measured either in millimicrons (mμ) or in Ångström units (Å). Ångström is pronounced "ongstrerm" and Å is the Swedish O. The following is a comparison of various units of length:

0.001 mm. = 1μ
0.000,001 mm. = 1mμ
0.000,000,1 mm. = 1 Å

Preparation of Solutions of Hematin Compounds:

Hemoglobin, by reducing oxyhemoglobin with sodium hydrosulfite.

Carboxyhemoglobin, by passing carbon monoxide through oxyhemoglobin solution.

Methemoglobin (acid), by adding ferricyanide to oxyhemoglobin.

Methemoglobin (alkaline), by adding alkali to acid methemoglobin.

Hematin (acid), by adding acetic acid to oxyhemoglobin, or by adding acetic acid to a solution of hematin in gum arabic.

Hematin (neutral or alkaline), by dissolving hematin * in water, or by dissolving hemin in dilute alkali.

Pyridine hemochromogen, by adding pyridine, sodium hydrosulfite, and sodium hydroxide to almost any hematin compound such as hemin, hematin, oxyhemoglobin, methemoglobin, etc.

Hematoporphyrin (acid) by dissolving hematoporphyrin in hydrochloric acid.

178. Cytochrome. Mix one cake of Fleischmann's yeast with about 80 ml. of water and fill a 1.5 × 6 × 10 cm. glass cell with the suspension. Using the large spectroscope and a very intense electric light, observe the absorption bands of reduced cytochromes a, b, and c. The a band, which is given by cytochrome a, will be difficult to see. The b and c bands, which are due to cytochromes b and c, will be plain. The d band, which is an aggregate of three bands given by the three cytochromes, will not be visible. The bands are located, respectively, at 603, 565, 550, and 527 mμ.

If it is convenient, ask the instructor to arrange to bubble a stream of air through the yeast suspension. Now observe the suspension with a spectroscope while air is bubbling through it. The bands will have disappeared since the cytochromes are oxidized. As soon as the stream of air is stopped, the bands will reappear.

179. Preparation of Cytochrome C (Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 [1937]). One beef heart is carefully freed from fat and ligaments and is ground several times in a meat grinder. Press the blood out of the ground material by hand by squeezing the material in cheesecloth. Mix 1100 g. of the pulp with 1100 ml. of 0.15 *N* (2½ per cent) trichloroacetic acid. Allow to stand at room temperature for two hours with occasional stirring. The pH of the mixture is now about 4. Press the liquid out by hand, using cheesecloth, and neutralize it to pH 7 with dilute sodium hydroxide.

* Prepared according to the method of Anson, M. L., and Mirsky, A. E *J. Gen. Physiol.* **13**, 469 (1930). See Exp. 181.

Centrifuge for 10 minutes. The clear supernatant so obtained shows strong absorption bands of reduced cytochrome c together with weak oxyhemoglobin bands.

To each 100 ml. of the supernatant add 50 g. of solid ammonium sulfate. Filter off the precipitate. The filtrate is not free from hemoglobin. To each 100 ml. of this filtrate add 5 g. of solid ammonium sulfate and leave in the ice chest over night. The pH of the mixture is about 4.9. The next day the precipitate is filtered off and the liquid, while still cold, is treated with 1/40 of its volume of 20 per cent trichloroacetic acid. This brings the pH of the mixture to 3.7. Within 10 minutes the spectrum of reduced cytochrome disappears and the cytochrome is completely precipitated in the oxidized form.

The cytochrome is centrifuged down and is shaken with 500 ml. of saturated ammonium sulfate solution. Centrifuge again. The red precipitate is transferred to cellophane tubing by means of about 20 ml. of distilled water and the mixture is dialyzed for 2 days against distilled water.

180. Preparation of Hemin (Schalfejeff method, modified by Nencki and Zaleski. Aberhalden's Handlexikon, vol. 6, 1911).

Place 500 ml. of glacial acetic acid and about 20 g. of sodium chloride in a 2 liter Erlenmeyer flask. Add with rotation from a pipette 100 ml. of oxalated blood (cow). Now heat over a flame to about 70° C. The formation of hemin crystals can be noted by rotating the liquid and observing the "silkiness." Now cool and then centrifuge off the hemin crystals and wash twice by stirring with acetic and hydrochloric acid (5 vols. glacial acetic and 2 vols. tenth normal hydrochloric) and then centrifuging. Stir with water and centrifuge. Repeat until all of the acetic acid is removed. Dry in an oven at 50° C.

The hemin may be recrystallized (Hogness, T. R., Zschiele, F. P., Sidwell, A. F., and Barron, E. S. G., *J. Biol. Chem.* **118**, 1 [1937]) by dissolving it in 25 ml. of a 4 per cent solution of quinine in chloroform. If quinine is not available you can use a 5 per cent solution of pyridine in chloroform. Warm gently if necessary. Do not boil. After the hemin is dissolved, filter. Add the hemin solution dropwise (from a separatory funnel) to 30 ml. of a saturated solution of NaCl in glacial acetic acid (in the hood). The acetic acid solution should be placed over a low flame while the hemin solution is added. Beware of fire (chloroform boils at 64° C., glacial acetic acid at 118° C.). Use a thermometer and do not let the temperature of the boiling mixture

rise above 90° C. After all of the chloroform is removed, allow to cool for 24 hours (in the hood). Observe the crystals under the microscope. Filter. Wash the hemin with dilute acetic acid, then with water, with alcohol and finally with ether.

These reagents and preparations are expensive. Be careful. Work quantitatively.

181. Preparation of Hematin. Add to 10 ml. of oxalated blood, 10 ml. of 0.1 *N* HCl and then 200 ml. of acetone which contains 2 ml. of *N* HCl. Mix and filter. Wash the residue of denatured globin with a little more acetone (two 10 ml. portions containing no HCl). Now add to the acetone solution of hemin one per cent of its volume of 2 *N* sodium acetate. This will precipitate hematin in crystalline form.

The preparation of hematin as described above is taken from the method of Anson and Mirsky (Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.* **13**, 469 [1930]). However, Anson and Mirsky employ hemoglobin solution instead of oxalated blood.

182. Preparation of an Acetone Solution of Hemin. Weigh out about 200 mg. of perfectly dry hemin and dissolve in acetone, plus about 1 ml. of concentrated HCl, diluting to 1000 ml. in a volumetric flask with acetone. Stopper and having placed in the flask a few glass beads, invert the flask and rotate to grind up the hemin until it dissolves entirely.

183. Determination of Iron in Hemoglobin. This method is a modification of the one by Saywell and Cunningham (*Ind. Eng. Chem., Anal. Ed.* **9**, 67 [1937]). It can be employed to determine iron in various biological materials. In this experiment the student is asked to determine the quantity of iron in 100 ml. of blood and to use this value to calculate the amount of hemoglobin present.

Pipette into a thin-walled digestion tube exactly 1 ml. of oxalated blood which previously has been diluted 10 times with water. Use a Folin-Ostwald pipette to do this. Add 1 ml. of concentrated sulfuric acid and about 8 drops of redistilled (iron-free) concentrated nitric acid. Heat gently until all of the organic matter has been oxidized and until the presence of white fumes of sulfur trioxide shows that all of the nitric acid has been driven off. It may be necessary to add more nitric acid and to heat again, if all of the organic matter has not been oxidized by the time that white fumes appear.

Remove the flame and allow the tube to cool well. Add 20 to 30 ml. of distilled water and transfer the solution quantitatively to a 100 ml. graduated flask, using a funnel and washing with distilled water.

Now add to the flask 1 ml. of the hydroxylamine solution and 1 ml. of the *o*-phenanthroline solution. Rotate the flask and add, drop by drop, concentrated ammonium hydroxide until the solution has become a light pink. Drop a small piece of Congo red paper into the flask. Now continue adding the ammonium hydroxide until the paper shows signs of turning pink. Now add dilute ammonium hydroxide, drop by drop, until the paper is pink. Dilute the solution to the 100 ml. mark and mix well. If the solution contains any precipitate it must be filtered, using iron-free filter paper. This can be prepared by washing filter paper with hydrochloric acid, then water, and then drying.

Using a blue glass filter and water in the tube, set the photoelectric colorimeter* at the null point. Now read the unknown and find from the standard graph how much iron the reading indicates.

The standard graph is prepared by reading various solutions prepared as described above, using 0.01 to 0.10 mg. of standard iron sulfate solution. This solution is made from "analytical" iron wire, free from rust. One weighs accurately 0.1 to 0.2 g. of this wire and dissolves it in 40 ml. of 10 per cent sulfuric acid and 1.5 ml. of redistilled, concentrated nitric acid. The solution is finally boiled gently until fumes of sulfur trioxide are given off. It is then cooled and diluted to 1 liter volume and mixed. The iron content per ml. is then calculated from the weight of wire employed and from the per cent of iron in the wire. This latter figure is supplied by the firm that supplies the wire.

Reagents:

1. Hydroxylamine hydrochloride. Use a 10 per cent solution in water.
2. *o*-Phenanthroline (The G. Frederick Smith Chemical Company, Columbus, Ohio). Use a 0.75 per cent solution in ethyl alcohol.
3. Sulfuric acid, concentrated, C.P.
4. Nitric acid, concentrated, iron-free, C.P.
5. Ammonium hydroxide, (a) concentrated, (b) concentrated diluted 1 to 5 with distilled water.
6. Congo red indicator paper.
7. Ferric sulfate standard solution.

AGGLUTINATION

184. Hemagglutination. Centrifuge some dog, horse, cat, rat, rabbit, or guinea pig oxalated blood and wash the erythrocytes three

*For example a Klett-Summerson colorimeter.

times by mixing with 0.9 per cent salt solution and centrifuging. The third centrifugation should be carried out in a graduated centrifuge tube, and the volume of the sedimented erythrocytes should be read. Now make a 2.5 per cent (by volume) suspension of these cells in 0.9 per cent salt. Pipette 1 ml. of the suspended cells into 5 different dry, clean test tubes. Add to the tubes various dilutions of concanavalin A, using 1 ml. each time. Mix at once and allow to stand at room temperature, observing from time to time, after shaking, to see if agglutination has occurred. It is most easy to detect agglutination by use of the microscope. It is well to compare a drop of untreated red cell suspension with the unknown.

The concanavalin A solutions are prepared by diluting 1 ml. of a 4 to 6 per cent solution of concanavalin A in saturated sodium chloride to 100 ml. with 0.9 per cent salt solution. Next 10 ml. of the diluted solution can be mixed with 90 ml. of 0.9 per cent salt and thus until 4 dilutions have been made.

(Concanavalin A and Hemagglutination. See Sumner, J. B., and Howell, S. F., *J. Immunology* **29**, 133 [1935]; Sumner, J. B., Howell, S. F., and Zeissig, A., *Science* **82**, 65 [1935]).

185. Does a Solution of Concanavalin A Agglutinate Washed Cow Erythrocytes? Add a drop of concanavalin A to 1–2 ml. of a solution of glycogen. What happens? Now add glycogen to a suspension of cow erythrocytes and again test with concanavalin A to see if agglutination occurs. Note that untreated cow erythrocytes are not agglutinated.

186. Does Concanavalin A Agglutinate a Suspension of Corn or Rice Starch? Yeast?

ENZYMES

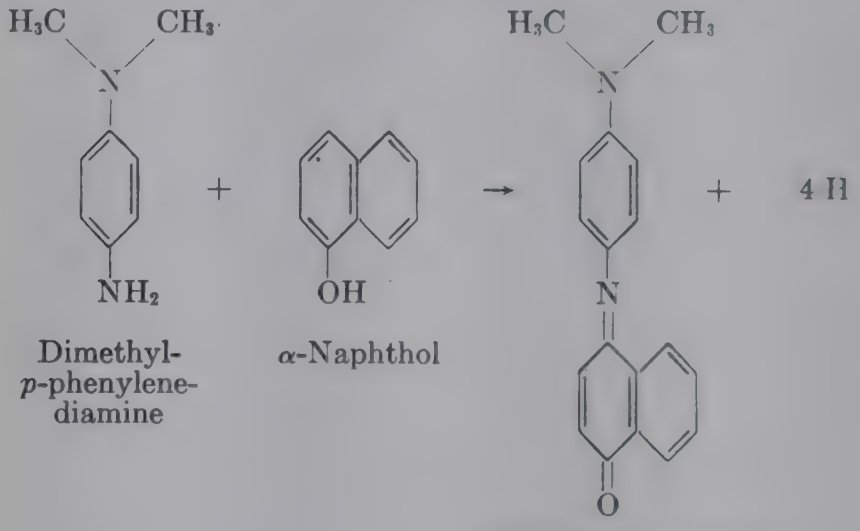
187. Tyrosinase, Catechol Oxidase. Stir a half-teaspoon of powdered acetone-dehydrated potato with 20 ml. of water and use this source of tyrosinase to make the following tests.

A. Boil a few crystals of tyrosine in 2 ml. of water to dissolve, cool, and add to 5 ml. of the tyrosinase. Observe after 2 hours to see if the material is colored red. This red substance is hallochrome. Observe after 24 to 48 hours to see if the color is black. The black substance is melanin.

B. Add about 1 ml. of a saturated aqueous solution of *p*-cresol to 5 ml. of the tyrosinase. Observe the production of an oxidation product.

C. Add 1 ml. of an aqueous solution of phenol to 5 ml. of the tyrosinase solution. Observe after one hour.

D. Add to 5 ml. of the tyrosinase solution about 5 drops of a fresh aqueous solution of dimethyl-*p*-phenylenediamine hydrochloride (0.2 per cent) and 5 drops of an alcoholic solution of α-naphthol (0.2 per cent). This mixture is called the "nadi" reagent from the words "naphthol" and "diamine." Observe the production of a blue color. This blue product is dimethyl indophenol blue. The reaction is:

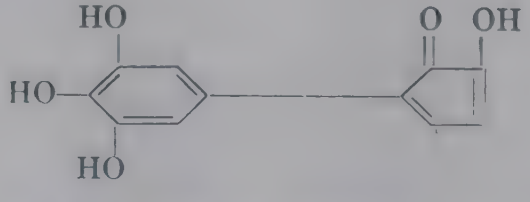


Dimethyl-*p*-phenylene-diamine

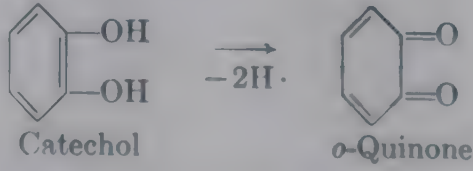
α-Naphthol

Dimethyl indophenol blue

E. Add 1 ml. of 3 per cent aqueous pyrogallol to 5 ml. of the tyrosinase solution. Observe the production of a yellow color. The yellow substance is purpurogallin.



F. Add 1 ml. of 1.0 per cent catechol solution to 5 ml. of the tyrosinase solution. Observe the production of a brown color. The catechol is oxidized to *o*-quinone.

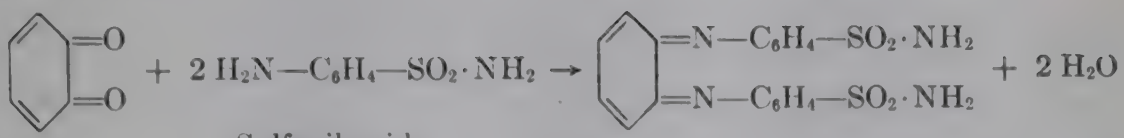


Catechol

o-Quinone

Now place one-half of the digest in a second test tube and add 2 ml. of a solution of sulfanilamide. Observe the production of a

red color.



Sulfanilamide

G. Add 2 drops of fresh alcoholic gum guaiac solution (2 per cent) to 5 ml. of water and add a pinch of acetonized potato. Little or no color will be produced. Now pour one-half of the digest into a

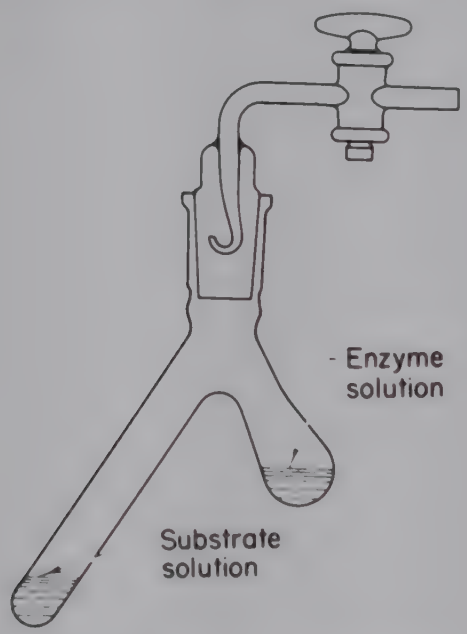


Fig. 15. Modified Thunberg tube.

second test tube and add 1 or 2 drops of a 0.003 per cent solution of catechol. Notice the blue color. Here the tyrosinase has oxidized the catechol to *o*-benzoquinone, and the quinone has oxidized the gum guaiac to a blue oxidation product.

The acetone-dehydrated potato is prepared by slicing whole potatoes with a knife board and letting the very thin slices drop immediately into a large beaker of acetone. Later, after stirring well, the acetone is poured off and more acetone is poured on. After stirring and standing, this is poured off and the material is dried on cheesecloth. The next day the material is ground powder-fine in a porcelain ball mill equipped with quartz pebbles.

188. Colloidal Platinum as Oxidation Catalyst. To 2 ml. of a suspension of colloidal platinum in gum arabic, add a few drops of freshly prepared 2 per cent alcoholic gum guaiac and mix. Note the blue color. The colloidal platinum acts as a catalyst in transferring

atmospheric oxygen to the guaiaconic acid, which is a constituent of the gum guaiac. The oxidation product of guaiaconic acid is blue.

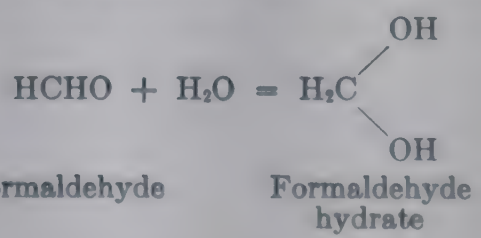
The colloidal platinum has been prepared by adding 1 ml. of a 10 per cent solution of chlorplatinic acid to 50 ml. of 2 per cent gum arabic. To this is added 2 ml. of 1 per cent formaldehyde and 1 ml. of 10 per cent sodium carbonate and the mixture is heated in boiling water for 20 minutes. The gum arabic acts as a protective colloid. Colloidal platinum, while a catalyst, is not an enzyme.

189. The Schardinger Enzyme, Xanthine Oxidase. Unheated milk contains an enzyme known as the Schardinger enzyme, xanthine oxidase, or aldehydrase. This is a dehydrogenase which removes hydrogen from hydrated aldehydes, hypoxanthine, and xanthine, provided a hydrogen acceptor is present.

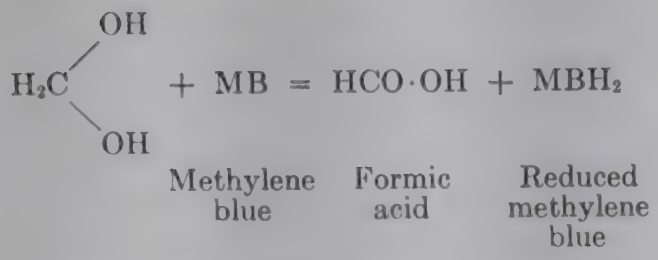
If a modified Thunberg tube is available, place 5 ml. of unpasteurized milk and 1 ml. of 0.02 per cent methylene blue in the tube and place 1 ml. of 0.4 per cent formaldehyde in the side bulb. Carefully evacuate the tube and fill it with nitrogen.* Again evacuate and fill with nitrogen. Now invert the tube so that the formaldehyde flows down into the tube. Mix and place the tube in a water bath at 37° C. Note the time needed for the blue color to disappear.

Before using a Thunberg tube, test it to see whether the stopper sticks or leaks. It is likely to do both. If it is filled with nitrogen it is less likely to leak than if it is left evacuated. The tube shown in Fig. 15 is a modification of a tube developed by Thunberg (*Quart. Rev. Biol.* **5**, 318 [1930]) for use in studying oxidizing enzymes. The modified tube can be constructed readily from stock ground joints and stopcocks. It is easier to use than the original tube and is less likely to leak.

If a Thunberg tube is not available, place 5 ml. of milk (unpasteurized) in a test tube and add about 1 ml. of 0.02 per cent methylene blue and 1 ml. of 0.4 per cent formaldehyde solution. Mix and cover with heavy mineral oil and keep at 37° C. in a water bath. Is the dye decolorized? How can one use controls for this experiment? What is the function of the mineral oil? The reaction is:



* If nitrogen is not available the tube may be left evacuated.



190. The Peroxidase-like Action of Hematin. Test 3 ml. of a greatly diluted solution of hemoglobin, or hematin, or hemin (an acetone solution of hemin diluted with water) by adding 0.5 to 1 ml. of benzidine solution and 1 drop of 3 per cent hydrogen peroxide. If one part of hemin per 6,000,000 is present, a blue color will be produced. The benzidine solution contains 2 g. benzidine, 10 ml. glacial acetic acid, 10 g. sodium acetate, 100 ml. alcohol, and 100 ml. water.

191. Peroxidase. To a pinch of jack bean meal add 3 ml. of water and about 5 drops of fresh alcoholic gum guaiac solution. Mix and add 1 drop of 3 per cent hydrogen peroxide. A blue color indicates the presence of peroxidase. Test for the presence of oxidase by omitting the addition of hydrogen peroxide. Is oxidase absent? Is peroxidase inactivated by heat? Boil a pinch of jack bean meal with 3 ml. of water, cool, and add gum guaiac solution and hydrogen peroxide.

192. Peroxidase. To a filtered extract of either horse radish root or ground, green barley or rye malt, add water and a few drops of saturated aqueous solution of ortho-cresol and then 1 drop of 3 per cent hydrogen peroxide. Repeat with meta-cresol. Repeat with para-cresol. Repeat with a solution of guaiacol.

Note: If too much hydrogen peroxide is added, the peroxidase will be inactive.

Preparation of Green Malt from Barley or Rye. Place 1 kg. of barley or rye in a large enameled pan. Add 650 ml. of water and mix well. Cover with another pan. Let stand for 3 days, mixing occasionally. Do not allow to dry out. By this time the barley or rye will be sprouted and the sprouts will be about one inch long. Now dry the material at room temperature by spreading out in a thin layer on clean towels. When well dried, grind the malt in a coffee mill.

193. The Catalytic Action of Colloidal Platinum on Hydrogen Peroxide. To 10 drops of colloidal platinum suspension add about 1 ml. of hydrogen peroxide. Note the rapid liberation of bubbles of

oxygen. Is this liberation increased by shaking? Repeat the above experiment after poisoning the colloidal platinum with 1 drop of 5 per cent sodium cyanide solution. Is the catalytic effect inhibited? When the experiment is finished, carefully pour the material into a bottle at the end desk for receiving waste cyanide.

194. Liver Catalase. To about 1 ml. of fresh, ground liver add about 2 ml. of 3 per cent hydrogen peroxide. Is there any doubt about the reaction?

Nearly all tissues (but especially blood and liver) contain the enzyme catalase, which decomposes hydrogen peroxide as follows:

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

Colloidal platinum has the same effect but is not an enzyme and is not destroyed by boiling. Both colloidal platinum and catalase are poisoned by cyanide.

195. Pepsin. Soak some dry carmine fibrin (Grützner, P., *Arch. ges. Physiol.* (*Pflügers*) **8**, 452 [1874]) with water and place enough in 3 test tubes to fill to about one-half inch. Label each test tube. To tube 1 add 3 ml. of pepsin solution and 3 ml. of water. To tube 2 add 3 ml. of pepsin solution and 3 ml. of tenth normal hydrochloric acid. To tube 3 add 3 ml. of pepsin solution and 3 ml. of 0.4 per cent acetic acid. (The pepsin solution can be made by adding 10 ml. of water to a quarter teaspoonful of Parke Davis or Fairchild's pepsin powder.) Mix all tubes well and observe from time to time, shaking to see if the red dye has been liberated by the digestion of the fibrin. This test must not be warmed, made alkaline, or allowed to stand over night, or the dye will be liberated in the absence of pepsin. One mg. of 1 : 3000 pepsin with hydrochloric acid will give a test in about 1 minute.

Preparation of Carmine Fibrin. Allow moistened fibrin to soak in a $\frac{1}{2}$ per cent solution of carmine (from cochineal) that contains a slight excess of ammonia (see note). When the fibrin will take up no more of the dye, wash thoroughly with water, then wash well with dilute acetic acid, and finally with water. Drain off the water and dry well in a thin layer on a towel.

Note: The carmine solution must be free from heavy metals. This purification can be accomplished by treating the carmine solution with hydrogen sulfide and filtering.

196. Plastein Formation. Dissolve 2.0 g. of Witte's peptone in about 6 ml. of distilled water. Now add 2 ml. of *N* HCl. Dilute to

10 ml. and mix. To one-half of the material add 2–3 drops of an approximately 10% pepsin (1 : 10,000) solution. Place both tubes in a pail of water at 50° C. and allow to stand. Several hours later (or next day) observe the gel of plastein which has formed in the tube which contained the pepsin.

The mere appearance of a precipitate upon adding pepsin means nothing. At pH 4.0, the addition of pepsin often causes a precipitate to form. This precipitate is composed of pepsin united with protein or proteose.

Note: Difco Bacto-Peptone is not suitable for use in this experiment.

197. Gastric Rennin (Rennet, or Chymosin). Place 3 ml. of fresh milk in 3 labelled test tubes. To tube 1 add 3 ml. of tenth normal hydrochloric acid. To tube 2 add a few drops of rennin solution (prepared by grinding up a junket tablet with 10 ml. of water). To tube 3 add a few drops of potassium oxalate solution and then a few drops of rennin solution. Mix and place tubes 2 and 3 in water at about 37° C. Is calcium necessary for the precipitation of paracasein? When milk is boiled, the calcium is precipitated. Boiled milk will not clot with rennin unless some calcium chloride is added. This is also true of solutions made from milk powder. The addition of acid to milk precipitates free casein.

Note: If rennin is not available, pepsin can be used instead.

198. Pancreatic Rennin. To 5 ml. of sodium caseinate just acid to methyl red, *i.e.*, pH 5.6; add a drop or two of trypsin, mix, and heat to 40°–60° C. A white precipitate will soon form. This is presumably some sort of paracasein, which is comparatively insoluble at this pH. The pancreatic rennin is presumably a trypsin; hence the above test can be employed for trypsin.

The sodium caseinate solution can be prepared by soaking 10 g. of casein in 100 ml. of water. Now one adds 56 ml. of 0.1 *N* sodium hydroxide. When the casein is nearly all dissolved, one adds 1 g. of potassium oxalate dissolved in 10 ml. of water, and then, with constant stirring, one adds about 32 ml. of 0.1 *N* hydrochloric acid, or enough to make the solution just red to methyl red.

The oxalate is added to remove calcium, which would, if present, allow the casein to be precipitated by ordinary rennin (gastric rennin) as calcium paracaseinate.

199. Trypsin. Prepare a solution of sodium caseinate as follows: To one-half g. of casein add 20 ml. of water and 1 ml. of phenol red.

Mix, then add 15 ml. of tenth normal sodium hydroxide. Mix well and warm to 35° C. Run in tenth normal hydrochloric acid with constant rotation until the solution is only slightly alkaline to phenol red. Dilute to 50 ml. volume.

Prepare a solution of trypsin by adding about 10 ml. of water to a quarter teaspoonful of Fairchild's trypsin powder and mix. Place 2 ml. of sodium caseinate in four labelled and numbered test tubes. Add to tube 1 one ml. of trypsin solution; to tube 2 five drops of tenth normal hydrochloric acid and 1 ml. of trypsin solution; to tube 3 five drops of tenth normal sodium hydroxide and 1 ml. of trypsin solution; to tube 4 nothing. Mix and let stand at room temperature for 15 minutes or at 37° C. for 4 minutes. Now add to each tube two drops of 10 per cent acetic acid. The undigested casein will be precipitated. If the casein has been entirely digested, the addition of acetic acid will produce no precipitate.

200. Hide-Powder-Barium-Sulfate Test for Trypsin (Sumner, J. B., and Howell, S. F., *J. Biol. Chem.* **109**, 429 [1935]). Place not more than 0.2 to 0.3 g. of dry hide powder-barium sulfate in a test tube and cover with water. After 5 to 10 minutes add a few drops of neutral phosphate buffer and enough water to bring the volume to about 7 ml. Now add about 1 ml. of *water-clear* trypsin solution and rotate the tube for 1 minute. Allow the hide powder to settle and observe whether the supernatant liquid is turbid. If not, wait for 10 minutes or longer, and shake the tube again, and again make observations.

The trypsin digests the hide powder (collagen and elastin) and sets free the barium sulfate. This reagent can be used to test qualitatively for papain-cysteine, bromelin-cysteine, ficin-cysteine, yeast protease, and malt protease, if the correct pH is obtained by adding the necessary buffer.

The hide powder-barium sulfate is prepared by adding 100 g. of hide powder (purchased from the Standard Manufacturing Co. of Ridgeway, Pa.) to 1000 ml. of 5 per cent boiling barium chloride. The flame is at once turned off and the material is stirred for 10 minutes. The moist material is now placed in cheesecloth on a funnel and the excess liquid is squeezed out. The material is next dumped into 1000 ml. of 10 per cent ammonium sulfate and stirred for 10 minutes. It is then placed on cheesecloth on the funnel and drained and squeezed. This washing is repeated 9 times more. The material is then spread out on a towel and allowed to dry. All lumps are broken up.

201. Test for Pancreatic Lipase (Sumner, J. B., unpublished). With rapid stirring add 10 ml. of 10 per cent castor oil in 95 per cent ethyl alcohol to 50 ml. of distilled water. Filter through a small piece of cotton to remove large droplets of fat. To 10 ml. of the fat suspension add about 1 ml. of alkaline buffer (1 vol. *N* NH_4OH + 1 vol. *N* NH_4Cl). To one-half of this add about 3 drops of pancreatic lipase. Keep the other one-half as a control. If the lipase is active the fat suspension will clear up in a short time because the fat will be entirely digested. Observe both tubes side by side, holding them away from the light.

For the preparation of lipase see Exp. No. 219.

202. Esterase. Place a little fresh ground liver, or liver extract, or else dried liver powder, in a test tube. Add 5 ml. of water and 3 drops of ethyl butyrate. Add 1 ml. of phenol red and then 2 drops of NH_4OH—NH_4Cl buffer (prepared by mixing equal volumes of 1 *N* NH_4OH and 1 *N* NH_4Cl). If this does not give a red color with the phenol red, add just enough of the buffer to give a red color (meaning that the reaction is alkaline). Does the red color fade? This is due to the liberation of butyric acid. Try the same test, using boiled liver.

203. Amylase-Liquefaction of Starch Gel (Sumner, J. B., unpublished). Place *potato* starch of the volume of ½ pea in a clean test tube. Add 2 ml. of distilled water. Place the tube in a beaker of boiling water and twirl rapidly until the starch has formed a clear gel that is just firm enough so that it will hardly flow out when the test tube is inverted. Remove and cool somewhat. Add 3 drops of pancreatic amylase. Mix. Does the gel liquify? If so the presence of amylase is proven. This is the most rapid test for amylase.

The student can obtain a gel of the proper consistency by suspending 2 g. of potato starch in 50 ml. of water and pipetting 2 ml. of this into a test tube before the starch has opportunity to settle out. The test tube is then heated, with shaking, over a flame or else in boiling water while being spun with the fingers.

204. Amylase. Test jack bean meal for amylase as follows: Boil a pinch of the meal with 5 ml. of water to dissolve the starch. Cool to 40°–50° C. and add a large pinch of fresh jack bean meal. Mix and place the tube in warm water. Remove a drop of the liquid from time to time and test for starch by adding iodine on a porcelain plate. Is the starch digested?

205. Pectase. (This is a repetition of Exp. No. 86. Pour 3 to 4 ml. of commercial pectin solution ("Certo") into a test tube. Add a few drops of phenol red and neutralize with tenth normal sodium

hydroxide. Add about 2 g. of jack bean meal and shake well. Is the pectin coagulated by the pectase present in the jack bean meal? Notice that the material becomes acid during the process of coagulation. This is due to the hydrolysis of the pectin to form pectic acid and methyl alcohol. The reaction has nothing to do with the gelation of pectin when cane sugar is added, as in the preparation of jelly.

Keep the tube containing the gel for several days and then note the phenomenon of syneresis; *i.e.*, contraction of the gel and exudation of serum.

206. Urease. Dissolve a few crystals of urea in 2 to 3 ml. of water and add a few drops of phenol red solution. Now add a pinch of jack bean meal. Note that the yellow color turns red almost instantly. The urease converts the urea to ammonium carbamate and this spontaneously changes to ammonium carbonate. Thus, the reaction becomes alkaline.

207. Enzyme Unknowns. Hand in two clean, dry test tubes for enzyme unknowns. These may contain any, all, or none of the following enzymes: pepsin, trypsin, lipase, amylase, urease, peroxidase.

For lipase use the test described in Exp. 201.

For amylase use the test described in Exp. 203.

For trypsin use the test described in Exp. 200.

For peroxidase use guaiacol and hydrogen peroxide as described in Exp. 192.

208. Inactivation of Urease by Lead, Silver, and Mercuric Ions (Sumner, J. B., and Myrbäck, K., *Z. physiol. Chem.* **189**, 218 [1930]).

To about 2 ml. of urease solution containing about 10 units per ml., add 5 drops of lead acetate and mix. Now add 5 drops of phenol red solution and a few crystals of urea. Is the urease active? Does it slowly decompose the urea?

To about 2 ml. of urease add 1 drop of *N*/1000 silver nitrate and mix. Now add phenol red and urea crystals. Has the urease been completely inactivated? Add 2 drops of water saturated with hydrogen sulfide and note the effect of removing silver as silver sulfide.

Pour some Nessler solution into a small test tube, then pour back into the bottle of Nessler solution. Now rinse the tube with tap water and with distilled water several times. Now add 1 ml. of urease solution, 5 drops of phenol red, and a few urea crystals and mix well. Have the walls of the tube become poisoned? Clean the test tube with potassium iodide solution and again test urease solution with urea and phenol red.

209. Determination of Urease Activity by Aeration (Sumner, J. B., and Hand, D. B., *J. Biol. Chem.* **76**, 149 [1928]).

In this experiment all glassware must be free from surface films of mercury, silver, or copper. Such films can be removed by the use of concentrated potassium iodide solution, concentrated nitric acid (for mercury), sodium thiosulfate followed by concentrated nitric acid (for silver) or concentrated nitric acid (for copper). Chromic acid in sulfuric acid, ordinarily spoken of as cleaning solution, must not be used, since some of it remains in the pores of the glass and poisons the urease later (Henry, R. J., and Smith, E. C., *Science* **104**, 426 [1946]).

Pipette into a clean, large test tube exactly 1 ml. of urease solution which has been previously diluted to contain between 0.5 and 1 units per ml. Place in a bath at 20° C. After 3 or more minutes add rapidly by pipette about 1 ml. of urea-phosphate solution which has been brought to 20° C. by standing in the bath in a test tube. Mix at once, noting time by stop watch. After 5 minutes blow into the digest 1 ml. of *N* hydrochloric acid. Add 2 more portions of the hydrochloric acid, mixing well. Add 15 drops of caprylic alcohol. Place in this tube the aeration apparatus. Next pipette into another large test tube 15 ml. of 0.01 *N* hydrochloric acid, 2 drops of 0.02 per cent methyl red, and 1 drop of neutral caprylic alcohol, and stopper with a receiving tube. Now add 1 ml. of saturated sodium hydroxide to the tube containing the digest and stopper at once. Connect up the aeration train and aerate slowly for 3 minutes and rapidly for 40 minutes. Now disconnect the receiving tube and titrate with 0.005 *N* barium hydroxide. Calculate the mg. of ammonia nitrogen formed per ml. of urease solution in 5 minutes at 20° C. This is equivalent to units of urease.

The phosphate solution is made by dissolving 28 g. anhydrous KH_2PO_4 and 68 g. anhydrous Na_2HPO_4 in about 900 ml. of redistilled water. This is then placed in a 1000 ml. flask and saturated with hydrogen sulfide in the hood. The next day it is filtered and boiled down to two-thirds its volume in a liter beaker. It should be filtered directly into boiling redistilled water and the boiling continued as fast as it filters through. When the boiling is over, cool, dilute to 1000 ml., and preserve with toluene.

To 100 ml. of this phosphate solution add 3 g. of purest urea and mix until dissolved. Add toluene and keep in ice chest when not in use. If the phosphates crystallize out, the solution, before it is used, must be warmed and mixed until they have all redissolved.

210. Determination of Urease Activity by Direct Nesslerization. If one has a solution of crystalline urease free from acetone or other interfering substances, it is possible to digest urea in phosphate buffer with this and to Nesslerize the digest directly and compare with a standard. See Exp. 209 for notes on cleaning glassware for this experiment.

Pipette into a tube graduated at 50 ml., 1 ml. of the sufficiently diluted urease solution and allow to come to 20° C. in a bath. Now add about 1 ml. of urea-phosphate solution at 20° C. and mix. After exactly 5 minutes, add 1 ml. of 1 *N* hydrochloric acid and mix at once. Dilute the contents of the tube to 50 ml. with distilled water and mix. Pipette an aliquot containing 0.1 to 0.3 mg. of ammonia nitrogen into a 200 ml. volumetric flask. Dilute until two-thirds full. Now Nesslerize using 10 ml. of Nessler solution. Dilute to mark, mix, and read in the photoelectric colorimeter using a blue glass filter. The mg. of ammonia nitrogen in the total digest will be equivalent to the units of urease in 1 ml. of the enzyme solution.

211. Digestion and Inactivation of Urease by Pepsin (Sumner, J. B., Kirk, J. S., and Howell, S. F., *J. Biol. Chem.* **98**, 543 [1932]).

Pipette into 2 large test tubes 1 ml. of crystalline urease solution containing about 600 units and add to each tube 2 ml. of acetate buffer at pH 4.3. Add to one tube 20 ml. of active pepsin (10 mg. of 1 : 10,000 pepsin) and to the other 20 ml. of boiled pepsin. Mix and place at once in a water bath at 40° C. At once remove 1 ml. samples from each tube and pipette each sample into labelled flasks containing 25 ml. of gum arabic-phosphate solution and mix. After 30, 60, and 90 minutes again remove samples and pipette into gum arabic-phosphate solution. Now run determinations of urease activity upon all samples.

Note that when the active pepsin is added to the urease in acetate buffer a precipitate forms and that this precipitate, which is composed of urease and pepsin, gradually dissolves as time elapses. At the same time that you remove samples for determination of urease activity, also remove 1 ml. samples from the solution containing active pepsin and place them in small test tubes. Add at once 5 ml. of dinitrosalicylic acid and mix. This reagent precipitates only urease and not pepsin in dilute solution. It is, therefore, possible to follow the digestion of the urease by observing the amount of urease that precipitates.

Plot a curve on coordinate paper, using as abscissae time in minutes and as ordinates the urease units per ml. of diluted digest.

The pH 4.3 acetate buffer is prepared by mixing 63 ml. of *N* sodium acetate with 137 ml. of *N* acetic acid. (Note that the sodium

acetate has 3 molecules of water of crystallization.)

The pepsin solution is made by diluting 10 ml. of 1 per cent Parke Davis, 1 : 10,000 pepsin to 200 ml. Boiled pepsin is prepared by heating some of this diluted pepsin for 10 minutes in boiling water in a large stoppered test tube.

The gum arabic-phosphate solution is made by heating 20 g. of powdered gum arabic with about 600 ml. of water until dissolved, adding 75 ml. of 9.6 per cent neutral phosphate and filtering. This filtrate is then diluted to 1 liter. To each 300 ml. of this one adds 10 ml. of neutral phosphate buffer solution.

212. Crystallization of Pepsin (Northrop, J. H., *J. Gen. Physiol.* **13**, 739 [1930]).

Weigh 100 g. of Parke Davis pepsin, 1 : 10,000, into a 1000 ml. beaker; add 100 ml. of distilled water, and stir gently until dissolved. Now add 100 ml. of *N* sulfuric acid. Add with stirring 200 ml. of magnesium sulfate, saturated at 20° C., and stir well. Filter through fluted paper (Schleicher and Schüll, No. 1450½). When the filtrate has ceased coming through, squeeze out the material upon a hardened filter paper on a Büchner funnel and continue filtering by suction. Wash the material twice with two-thirds saturated magnesium sulfate. Now, when the material has been sucked nearly dry, transfer to a 100 ml. beaker and stir with water to a thick paste. Now adjust a motor with glass stirrer and stir gently. From a pipette with a fine opening run into the stirred material 0.5 *N* sodium hydroxide. Great caution is necessary to avoid adding too much. Add the sodium hydroxide until the pepsin has dissolved. The liquid must always remain acid to Congo red paper.

Now cautiously add 0.5 *N* sulfuric acid until a heavy precipitate forms and leave the material in an ice chest for 3–6 hours in a place removed from the cooling unit. Filter with suction.

Now place the material in a 100 ml. beaker and stir to a thick paste with water. Stir with the glass rod attached to motor stirrer, holding the beaker in a bath of water at about 45° C. Now add 0.5 *N* sodium hydroxide very cautiously until most of the material has dissolved. This is the crucial part of the experiment. It is better to add too little alkali than too much. The presence of denatured pepsin in the material is likely to lead one to think that not all the pepsin has been dissolved and thus one continues to add alkali when enough has already been added.

Now place the material in a stoppered Erlenmeyer flask and

suspend in a bucket of water at 45° C. Allow to remain for 24 hours. Examine for pepsin crystals under the microscope. Keep the material in the ice chest labelled with your name.

213. Determination of Peptic Activity (Anson, M. L., *J. Gen. Physiol.* **22**, 79 [1938]. See also, Northrop, J. H., Crystalline Enzymes, Columbia University Press, p. 152 [1939]).

Four ml. of 2.5 per cent hemoglobin solution are pipetted into a large test tube. Add 1 ml. of 0.3 *N* hydrochloric acid. The final pH is 1.6. Place the tube and contents in a water bath at 25° C. and allow to come to the temperature of the bath. Add 1 ml. of the enzyme solution, noting the time, and mix at once. After 10 minutes, add 10 ml. of 0.3 *N* trichloroacetic acid (estimated by titration) and shake the tube vigorously. Filter the suspension. Whatman No. 3 filter paper is recommended.

Pipette 5 ml. of the digestion filtrate into a 50 ml. volumetric flask (a graduated digestion tube may be used) and add 10 ml. of 0.5 *N* sodium hydroxide. Next add 3 ml. of the phenol reagent. Since the color formed depends somewhat on the rate at which the phenol reagent is added, this rate is standardized by adding the reagent as rapidly as is possible, still allowing the reagent to come out of the pipette as drops. Read in the colorimeter against a standard after 2 to 10 minutes.

The standard should be prepared as nearly as possible at the same time. Use 5 ml. of the dilute standard tyrosine solution and develop the color in the same way as with the digestion filtrate. Allow five minutes for the color to develop.

A blank should be run as follows: Mix 10 ml. of 0.3 *N* trichloroacetic acid with 4 ml. of the 2.5 per cent hemoglobin solution and 1 ml. of 0.3 *N* hydrochloric acid. Add 1 ml. of the enzyme solution and mix again. Filter. Pipette 5 ml. of the filtrate into a 50 ml. flask, add 1 ml. of the concentrated tyrosine solution and develop the color as above. Read against the standard after 5 minutes.

The intensity of color, or "color value" in each of these solutions is calculated in terms of milliequivalents of tyrosine. The color value of 5 ml. of digestion filtrate in milliequivalents is:

$$\frac{\text{reading for standard}}{\text{reading for digestion filtrate}} \times 0.0008$$

The color value of 5 ml. blank filtrate is:

$$\frac{\text{reading for standard}}{\text{reading for blank filtrate + added tyrosine}} \times 0.0008 \times \frac{19}{18} - 0.0008$$

Unless color-producing products are added with the enzyme, the blank is usually about 0.00008 milliequivalents of tyrosine. The color value of the digestion products present in 5 ml. of digestion filtrate is equal to the difference between the color value of 5 ml. of digestion filtrate and the color value of 5 ml. of blank filtrate. The number of enzyme units corresponding to the color value of the digestion products in 5 ml. of digestion filtrate may be calculated using the following equation, provided the color value is between 6×10^{-4} and 11×10^{-4} milliequivalents of tyrosine:

$$\text{Pepsin units} = \frac{\text{millequivalents of tyrosine} \times 10^4 - 1.70}{1.16 \times 10^4}$$

Tyrosine solutions: Prepare a solution containing 8×10^{-4} milliequivalents of tyrosine per ml. Use 0.1 *N* hydrochloric acid as the solvent. Determine the tyrosine content of the solution by use of the Kjeldahl method. This is the concentrated tyrosine solution and is used in estimating the blank.

The dilute standard tyrosine solution is prepared by diluting the above solution so that it contains 8×10^{-4} milliequivalents of tyrosine per 5 ml. Add sufficient hydrochloric acid to make the solution 0.2 *N*. Both of these tyrosine solutions are preserved by adding sufficient formaldehyde to make the solutions 0.5 per cent.

Phenol reagent: The phenol reagent prepared according to Folin and Ciocalteu (see Exp. 125), is diluted with twice its volume of water.

Preparation of hemoglobin: "Whipped beef blood is centrifuged 20 to 30 minutes. The serum and white corpuscles, which form a thin layer on top of the red corpuscles, are siphoned off and the red corpuscles are then mixed with an equal volume of cold 1 per cent sodium chloride solution and after centrifugation the supernatant solution is siphoned off again and the corpuscles are either stored frozen or dialyzed immediately and then stored frozen. The corpuscles are largely freed of color-producing substances not precipitable by trichloroacetic acid by dialysis in DuPont cellophane tubing 3/4 inches diameter." A marble is placed in each tube. "The tubes are placed in a tall vessel. Cold tap water is run into the lower part of the vessel at a rate sufficient to cause stirring. Occasionally the tubes are inverted and the hemoglobin solution is thus stirred by the marbles. After 24 hours dialysis the hemoglobin solutions from all the cellophane tubes are mixed and the mixed solution is stored frozen in small aluminum containers or cardboard ice-cream containers. It is easily possible to prepare enough dialyzed hemoglobin at one time for

thousands of proteinase estimations.

"To estimate the concentration of proteins in the dialyzed corpuscles a 3 to 5 g. sample is weighed out in a porcelain evaporating dish, and dried overnight at 105° C., and the dry weight is recorded. The number of grams of protein per ml. of sample is:

$$\frac{\text{weight of dried protein}}{(\text{weight of sample} - \text{weight of dried protein}) + 0.73\ \text{weight of dried protein}}$$

Preparation of hemoglobin substrate: "The dialyzed hemoglobin solution is diluted with water to give a solution containing 2.5 g. protein per 100 ml. and is centrifuged. The small precipitate is rejected. One mg. Merthiolate (Lilly) per 40 ml. is added as a preservative. Toluol should not be used, nor should the amount of Merthiolate be increased, since larger amounts give significant color with the phenol reagent. The 2.5 per cent hemoglobin solution is stored at 5° C.", Anson, M. L., *J. Gen. Physiol.* **22**, 79 (1938).

214. Absorption of Peptic Activity and Pepsin Protein upon Denatured Ovalbumin (Sumner, J. B., *Proc. Soc. Exptl. Biol. Med.* **31**, 204 [1933]).

Prepare a solution of crystalline pepsin by mixing about 20 mg. of pepsin crystals with 50 ml. water and 10 ml. of acetate buffer at pH 4.8, then diluting to 200 ml. and filtering. Measure out 100 ml. of this pepsin solution and pipette into it 5 ml. of well-suspended, coagulated ovalbumin. Rotate for 5 minutes and then filter. Determine the peptic activity and the pepsin protein in aliquots of this filtrate. Now take a measured amount of the remaining filtrate (use as much as you have left), and add 5 ml. of suspended ovalbumin, and rotate 6 minutes. Filter and again analyze aliquots of the filtrate for peptic activity and for pepsin protein. Repeat twice more. Now calculate the percentage of peptic activity and of pepsin protein removed by each absorption. Determine also the activity of the original pepsin solution.

Peptic activity can be determined by the method of Anson.

Pepsin protein can be determined by adding to 5 ml. of the clear filtrate 1 drop of 10 per cent sodium hydroxide, mixing, and then adding 1 ml. of 0.5 *N* sulfuric acid and mixing. The denatured pepsin is then compared in a nephelometer with suspensions similarly prepared, using the original pepsin solution at various dilutions.

The pH 4.8 acetate buffer is made by adding 180 ml. of *N* sodium acetate to 120 ml. of *N* acetic acid.

The ovalbumin is crystallized from fresh egg white and is once

recrystallized. The crystals are centrifuged off and dialyzed against distilled water until ammonia-free. The albumin content in the solution is determined by the Kjeldahl method. A solution is then prepared containing 1000 mg. of ovalbumin in 90 ml. This is heated in a stoppered flask in boiling water for 15 min. It is then cooled and to it is added 10 ml. of the pH 4.8 acetate buffer.

215. Preparation of Purified Trypsin by Reversible Heat Denaturation (Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.* **17**, 151 [1933]).

Stir up 1 g. of Fairchild's trypsin with 25 ml. of 0.1 *N* hydrochloric acid and heat for one minute by rotating the flask in a water bath at 80° C. Now cool rapidly in icewater. After 10 minutes add 6 g. of solid ammonium sulfate and stir until dissolved. Filter by gravity. To each 10 ml. of filtrate add 2 g. of solid ammonium sulfate. Stir until dissolved. Centrifuge off the precipitate and discard the supernatant liquid. Remove as much mother liquor from the centrifuge tube as possible by means of a rolled filter paper. Now dissolve the precipitate in enough 0.0005 *N* hydrochloric acid to make the final volume 25 ml. This solution will contain 0.01 trypsin units (Northrop) per ml. and will require diluting 1–10 for estimation of its tryptic activity by the method of Anson.

This method is based upon the method of Northrop and Kunitz for the preparation of crystalline trypsin (Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.* **16**, 267 [1932]). Do not heat a larger volume than 25 ml. to 80° C.

216. Determination of Tryptic Activity. (Anson, M. L., *J. Gen. Physiol.* **22**, 79 [1938]. See also, Northrop, J. H., Crystalline Enzymes, Columbia University Press, p. 155 [1939].)

The procedure for the determination of tryptic activity is the same as that for pepsin (see Exp. 213), with three exceptions. In the first place the substrate is denatured hemoglobin-urea-phosphate solution. Second, no hydrochloric acid is added to the substrate. In the third place, because of the urea in the substrate solution, it is necessary to wait 30 minutes, after adding the trichloroacetic acid, before filtering. This latter precaution must be taken both with the digestion mixture and the blank.

Color values are calculated in terms of milliequivalents of tyrosine as in Exp. 213. The curve in Fig. 16 is consulted for determining the enzyme units. Thus, if 5 ml. of the trichloroacetic acid filtrate contains 2×10^{-4} milliequivalents of tyrosine, 1 ml. of the enzyme solution contains 1.2×10^{-4} units of trypsin.

Hemoglobin substrate: The hemoglobin is prepared as in Exp. 213. "A solution is prepared containing 8 ml. of 1 *N* sodium hydroxide, 72 ml. water, 36 g. urea, and 10 ml. of 22 per cent hemoglobin (22 g. hemoglobin per 100 ml. solution). This alkaline solution is kept at

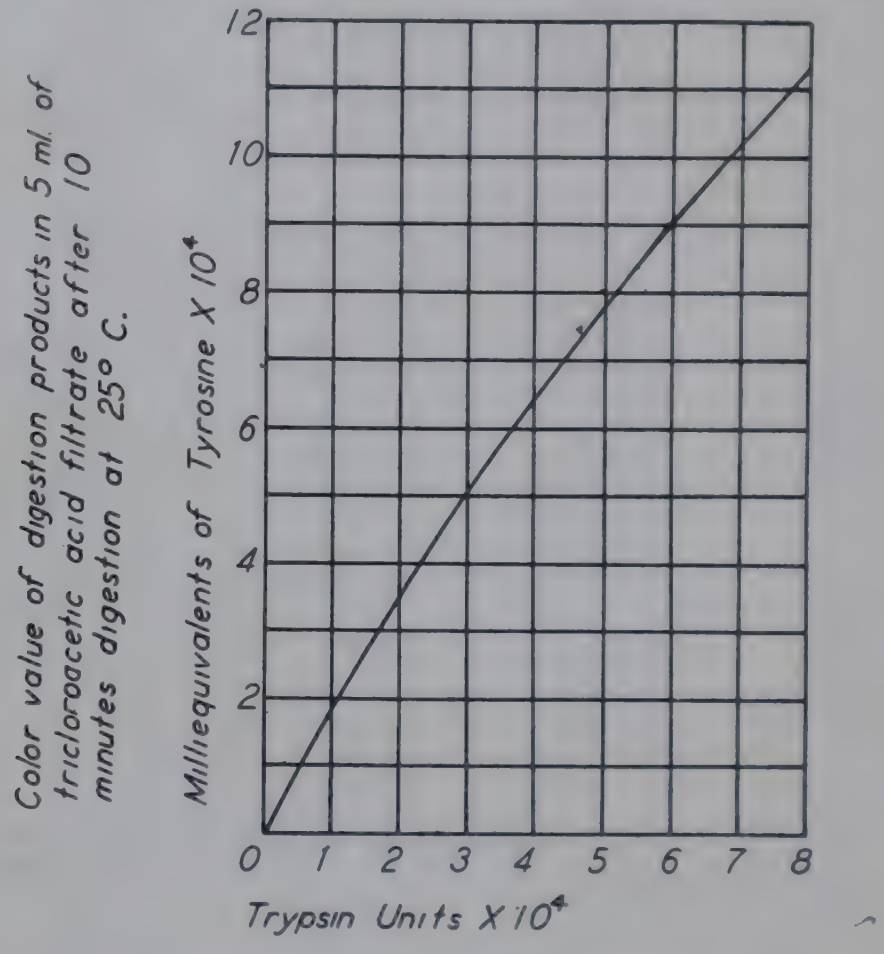


FIG. 16. Trypsin activity curve (Anson).

25° C. for 30 to 60 minutes to denature the hemoglobin and is then mixed with a solution containing 10 ml. 1 *M* potassium di-hydrogen phosphate and 4 g. of urea. The final pH is 7.5. One mg. Merthiolate (Lilly) is added to each 50 ml. of hemoglobin solution as a preservative, and the hemoglobin solution is stored at 5° C.," Anson, M. L., *J. Gen. Physiol.* **22**, 79 (1938).

217. Preparation of Powdered Pancreas (Willstätter, R., and Waldschmidt-Leitz, E., *Z. physiol. Chem.* **125**, 132 [1922]).

Obtain two dozen hog pancreases from a slaughter-house immediately after the hogs are slaughtered. Place in a clean, enamelled pail with ice and transport to the laboratory. Dissect off as much of the fat as possible, using scissors, and grind the remaining material with an ordinary household meat chopper. Put the material through the chopper 4 times, using a clean evaporating dish to hold the material.

Weigh the material and place it in a large, wide-mouthed bottle. Now add, with stirring, 1.82 l. of redistilled acetone to every kilogram of material. Allow to stand for 2 hours with occasional stirring. Filter by suction, using a hardened filter paper that has been wet with water and sucked down and then washed with acetone.

Repeat the treatment with acetone. Then employ one-half acetone with one-half ether. The ether must be freshly redistilled and entirely free from peroxide, as shown on testing with KI and boiled starch.

Defat twice in the above manner, using pure ether, 1.82 liters for every kg. of material started with.

Spread the material out to dry on filter paper on a towel in a dark room. Let it dry overnight.

Grind the material for several hours in a clean porcelain ball mill with quartz pebbles. It must be ground dust-fine. Get a large, clean, wide-mouthed bottle and place in it a piece of silk bolting cloth so that most of the cloth sticks out of the bottle. Place the powder on the cloth inside the bottle. Stopper and shake until no more powder will sift through. Keep this pancreas powder in a glass-stoppered bottle in a dark place.

218. Preparation of Enterokinase Powder (Waldschmidt-Leitz, E., *Z. physiol. Chem.*, **142**, 217 [1924–25]). Obtain about 24 first-meters of hog duodenum (from stomach down) from a slaughter-house immediately after hogs have been slaughtered. Place in a clean, enamelled pail with ice and transport to the laboratory. One should have a smooth board, 1 ft. × 6 ft., which has been covered on one side with hot paraffin. Spread an intestine out on the paraffined surface and open it out by cutting lengthwise with sharp scissors. With a clean glass plate, about 3 in. × 4 in., very gently scrape off the mucous coat. One must be careful not to scrape off any more than the slime. Continue thus with all intestines, adding the scrapings to a weighed liter beaker. (About 104 g. of material will be obtained from 10 meters of intestine.) When this is finished, weigh the beaker and calculate the amount of material. For every 100 g. add, with stirring, 300 ml. of acetone. Let stand 2 hours and filter with suction on a hardened filter. Repeat with acetone, then with half-acetone and half-ether, then twice with ether. Dry on filter paper overnight and grind in a ball mill. Sift through silk bolting cloth. Yield: 14 g. from 10 meters of intestine.

219. Preparation of Trypsinogen, Amylase and Lipase (Willstätter, *et al.*, *Z. physiol. Chem.*, **125**, 132 [1922].)

Dilute 870 g. of Kahlbaum's double-distilled glycerol (in dark

bottles) with water until the volume is 1 l. and mix. Weigh out 10 g. of powdered pancreas and add 100 g. of the 87 per cent glycerol. Stir well with a glass rod to break up all lumps. Place in a stoppered Erlenmeyer flask and keep in water bath at 30° for 4 hours, with occasional rotating to keep mixed.

Place in capped centrifuge tubes and centrifuge at 2500 revolutions for about $1\frac{1}{2}$ hours. Decant the supernatant liquid into a 100 ml. graduate and read the volume. For every 100 ml. add 200 ml. of water redistilled through glass and mix. Keep in a stoppered Erlenmeyer flask in the ice-chest. Add toluene.

To activate the trypsinogen to trypsin one adds 10 ml. of enterokinase to 10 ml. of the glycerol extract, mixes, and keeps in a bath at 30° for 30 min. The activated trypsin should be kept in the ice-chest. It does not keep as well as the unactivated trypsin.

220. Preparation of Enterokinase Solution. Weigh out 2g. of intestinal epithelium powder (from Exp. 218) and add to it 100 ml. of 0.04 *N* ammonium hydroxide. Stir well, adding the ammonia in small quantities at a time. Transfer to an Erlenmeyer flask, stopper, and place in a water bath at 30° C. and allow to stand for 3 hours with occasional rotating to mix. Place in centrifuge tubes covered with caps and centrifuge for one-half hour. Carefully decant the supernatant liquid into a 100 ml. graduate and measure the volume.

Pour the liquid into a 2500 ml. clean glass bottle. Blow into the bottle a blast of filtered air. The air can be filtered through cotton and delivered by a 14 inch glass tube which is bent downward towards the surface of the liquid. The bottle should be resting on its side. The air blast must be heated to about 30° by warming the farther end of the glass tube with a fantail burner.

Instead of this arrangement the Faust-Heim apparatus can be used, or else one can place the solution in an evaporating dish and blow air over it with an electric fan. The air blast is continued until the liquid has become evaporated to one-half volume. The material is then placed in a stoppered flask and kept in the ice-chest. It will keep for about 8 days.

The enterokinase can be further purified by adding to each 50 ml. 3.5 ml. of normal acetic acid with stirring. This is centrifuged and the clear supernatant fluid is poured with stirring into 700 ml. of redistilled (through glass) 95 per cent ethyl alcohol. The alcohol should be ice-cold. The material is kept in the ice-chest for an hour and then centrifuged. The tubes are drained well and the precipitate is dissolved in 30 ml. of water containing 1 ml. of 0.5 *N* phosphate at

pH 7.0 for every 50 ml. of solution taken at the start.

221. Determination of Tryptic Activity by Method of Willstätter, *et al.* (Willstätter, R., Waldschmidt-Leitz, E., Dunaiturria, S., and Kunstner, G., *Z. physiol. Chem.* **161**, 191 [1926]).

Estimation: Place in a 25 ml. flask with ground glass stopper, 5 ml. of 6 per cent neutral Kahlbaum casein, 2 ml. of NH_4OH—NH_4Cl buffer of pH 8.6 at 20°, or 8.9 at 30°, and 2 ml. of water. Let this come to the temperature of the bath. Now add 1 ml. of the sufficiently diluted trypsin and mix.

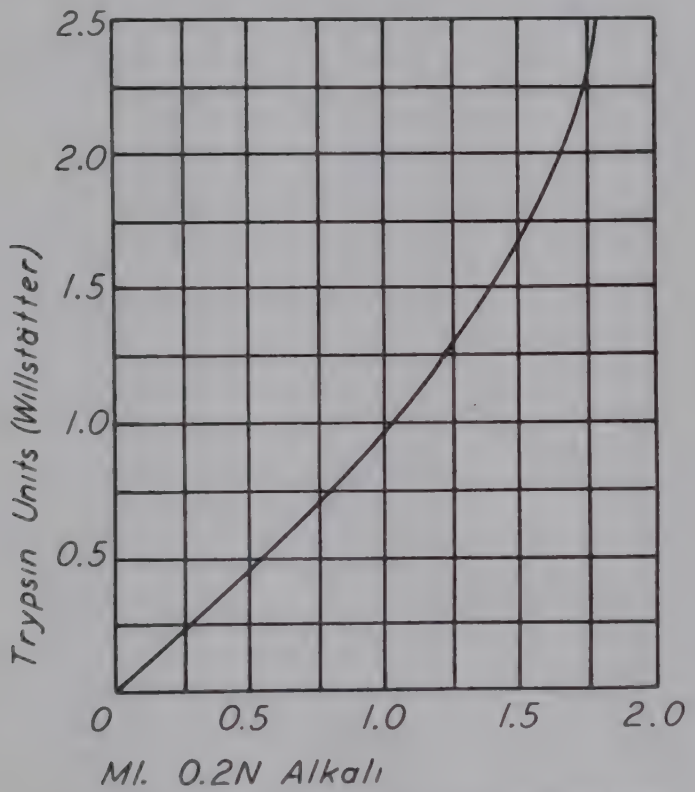


FIG. 17. Trypsin activity curve (Willstätter).

Allow to remain at 30° for 20 minutes. Using 5 ml. of water and 15 ml. of alcohol, rinse the contents of the 25 ml. flask into a 250 ml. Erlenmeyer flask. Add 5 ml. of 0.5 per cent neutral thymolphthalein in alcohol and titrate at once to a light blue with 0.2 *N* NaOH in 90 per cent alcohol. Now add 120 ml. of boiling absolute alcohol and titrate to a pale green, comparing with the standard suggested by Sumner (water, alcohol, borax, thymolphthalein, quartz powder, and enough HCl to give a green color).

Now run a blank using the same ingredients, but adding the trypsin just before titration. Subtract the blank. The difference is in ml. of 0.2 *N* NaOH. One trypsin unit requires 1.05 ml. of 0.2 *N* NaOH. Look up the digestion curve (Fig. 17) where ml. of 0.2 *N*

NaOH are plotted against the trypsin units. Calculate how many units of trypsin are present per ml. of the unknown.

To make the casein solution, add to 60 g. of casein about 70 ml. of 0.45 *N* NaOH from sodium. It should be about pH 7.0 when diluted to 1000 ml. The casein should soak in water for 30 minutes before adding the alkali. Preserve with toluene.

The NH_4OH—NH_4Cl buffer is 1 molar with respect to each constituent.

The NaOH in 90 per cent alcohol can be standardized by titrating against 0.05 *N* potassium acid phthalate.

222. Preparation of Saccharase from Yeast (Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, Washington, D. C., page 183 [1925]).

Five students are asked to work together.

Mix 1 lb. of Fleischmann's yeast with 500 ml. of water and then add 200 ml. of toluene. Stir often the first 24 hours. Let stand at room temperature for 7 days, stirring occasionally. Mix with Celite and filter through a folded filter paper. Mix the residue with 200 ml. of water and filter this. Combine both filtrates.

223. Determination of Saccharase Activity (Willstätter, R. and Kuhn, R., *Ber. Chem. Ges.*, **56**, 68 [1923]; Willstätter, R. and Roche, F., *Ann. Chem.* **425**, 1 [1921–22]).

Two students are asked to work together.

Dissolve 40 g. of purest sucrose in 0.1 *N* acetate buffer of pH 4.5 and dilute to 250 ml. volume in a 250 ml. volumetric flask with the buffer. Mix well and add a few drops of toluene. Now pipette out 50 ml. and read the rotation in the polariscope. Place the flask containing the remaining solution in a thermostat bath at 20° C. When this has been in the bath for about 10 minutes add to it 1 ml. of commercial saccharase and mix well, noting the time. After 30, 40, 50, and 60 minutes pipette out 25 ml. samples and place them at once in Erlenmeyer flasks containing 0.2 g. of sodium carbonate. Mix to stop the action of the enzyme and to mutarotate the invert sugar. Read each sample in the polariscope. Plot your rotations on coordinate paper against time in minutes and find just how many minutes it took to give the zero rotation. This is the time value of the saccharase. One saccharase unit has a time value of 1.

224. Determination of Saccharase Activity by Method of Sumner and Howell (Sumner, J. B. and Howell, S. F., *J. Biol. Chem.* **108**, 51 [1935]).

Place 5 ml. of sucrose-buffer in a test tube and allow to come to 20° C. in a thermostat bath. Add 1 ml. of saccharase, or yeast suspension, at 20° C. using a rapidly-delivering Folin-Ostwald pipette. Mix at once. After 5 minutes add 5 ml. of approximately 0.1 *N* sodium hydroxide and mix well. Determine the sugar in a 1 ml. aliquot, using the dinitrosalicylic method of Sumner, Experiment No. 50 (Sumner, J. B., *J. Biol. Chem.* **65**, 393 [1925]). If more than 1 mg. per ml. of invert sugar is present, or less than 0.5 mg., the analysis will have to be repeated, using a different concentration of saccharase, or else a different time interval. To obtain the saccharase units multiply the mg. invert sugar per ml. by eleven.

Sucrose-Buffer Solution: Place 43 ml. of *N* sodium acetate and 57 ml. of *N* acetic acid in a liter volumetric flask. Dilute to mark and mix. Dissolve 6.5 g. of C.P. sucrose in 96 ml. of the buffer. This will make a 6.5 per cent sucrose solution of pH 4.5. The solution will remain good for about 1 day at room temperature.

Table Showing Saccharase Concentrations

Source of Saccharase	*Sumner and Howell Units*
Fleischmann's yeast	994 per g. dry wt.
Nectar Brewing Co. yeast	830 " " " "
Taka diastase	48 " " " "
"Difco" glycerol invertase	3100 " ml.

225. Carbonic Anhydrase (Roughton, F. W. J., Ergebnisse der Enzymforschung **3**, 289 [1934] see p. 292 for a diagram of the apparatus).

Prepare 50 ml. of a solution containing *M*/5 Na_2HPO_4 and *M*/5 KH_2PO_4. Prepare a solution containing *M*/5 $NaHCO_3$ in *M*/50 sodium hydroxide.

Pipette into one side of the boat of the apparatus for carbonic anhydrase 2 ml. of the phosphate solution and 1 ml. of blood diluted 1–1000. Pipette into the other side of the boat 2 ml. of the sodium bicarbonate solution in sodium hydroxide. Immerse the boat in the thermostat bath at 20° C. and allow to come to this temperature. Bring the manometer to equilibrium by opening the upper stopcock and then closing it. Read the manometer. Now mix the contents of the boat, starting stop-watch at the same instant. Make manometer readings at 5, 10, 15, 30, 45, 60, 90, and 120 seconds, all the while shaking the boat under water at an even rate.

Now repeat this experiment, but use 1 ml. of water instead of 1 ml. of blood. Plot the two curves on coordinate paper, using ml. of carbon dioxide evolved as ordinates and seconds as abscissae.

226. Demonstration of Lactic Dehydrogenase, Diaphorase, and Coenzyme 1. Prepare five modified Thunberg tubes (Fig. 15, Exp. 189) by adding to each the following:

	Ml. to be added to tube no.				
Place in the tube:	1	2	3	4	5
Diaphorase suspension	1.0	1.0	1.0	—	1.0
Lactic apodehydrogenase	0.1	0.1	—	0.1	0.1
Methylene blue (1–5000)	0.1	0.1	0.1	0.1	0.1
Sodium lactate (5%)	0.1	—	0.1	0.1	0.1
HCN (1 *N*, see note)	1.0	1.0	1.0	1.0	1.0
Place in the side bulb:					
Coenzyme 1 (10 γ)	0.5	0.5	0.5	0.5	—

Whenever any of the reagents are not added to any of the tubes, or side bulb, an equal volume of water should be added so that the final volume is the same in all cases.

Note: The hydrocyanic acid must be delivered from a pipette which is filled by immersion. It must *never* be sucked up into a pipette by mouth.

Evacuate all of the tubes and fill them with oxygen-free nitrogen. Repeat the evacuation and fill once more with nitrogen. If nitrogen is not available, leave the tubes evacuated. Close the stopcocks and place the tubes in a water bath (with glass side) at 37° C. for 10 minutes. Mix the contents of the side bulbs with that of the tubes and place the tubes in the water bath again. Note the time. Observe for changes in the intensity of color of the methylene blue. Record the time when the methylene blue is completely decolorized.

If Thunberg tubes are not available use the alternative method described in Exp. 189.

See Exp. 244 for the preparation of coenzyme 1. The solution used for this experiment should contain about 20 γ per ml.

The lactic apodehydrogenase forms lactic dehydrogenase with the coenzyme 1. This removes hydrogen from the lactate, forming sodium pyruvate. The diaphorase removes the hydrogen from the reduced coenzyme 1 and gives it to the methylene blue, forming reduced methylene blue. The hydrocyanic acid unites with the pyruvate and, by removing this, prevents the reaction:

$$\text{Lactate} \rightleftharpoons \text{Pyruvate} + 2\text{H}$$

from coming to its equilibrium point.

The lactic apodehydrogenase is prepared by removing most of the visible fat from a fresh beef heart, cutting up the heart and grinding it coarsely in a meat chopper. This material, mixed with water, is ground in several portions in a Waring blender. Each time the blender should contain about 50 g. of heart muscle and 200 ml. of water. A little toluene is added. The preparation is filtered in the cold. (The residue is saved for the preparation of diaphorase.) To every 100 ml. of filtrate one adds 46 g. of solid ammonium sulfate and stirs gently until it is dissolved. The precipitated lactic apodehydrogenase is filtered off in the cold. The precipitate is scraped from the filter paper, dissolved in 500 ml. of water and again salted out by adding 46 g. of solid ammonium sulfate for every 100 ml. The apodehydrogenase is filtered in the cold, scraped from the filter paper and mixed with about 40 ml. of water and a little toluene and dialyzed overnight in the cold against distilled water. The next day the apodehydrogenase is transferred to a bottle and kept in the ice chest. It will remain serviceable for about 6 months.

The hydrocyanic acid is prepared by dissolving 6.0 g. of reagent grade potassium cyanide in about 250 ml. of water (in the hood). Add 2 drops of phenol red indicator and then, cautiously, add about 12.5 ml. of 7.5 *N* sulfuric acid, or enough to bring the solution to neutrality. The volume is now diluted to 300 ml. This solution of hydrocyanic acid is kept in the ice chest in a well labelled, well stoppered bottle.

The diaphorase is prepared from the insoluble residue left after filtering off the lactic apodehydrogenase. This material is suspended in about 2 liters of *M*/10 phosphate buffer, pH 7.0, and ground in the Waring blender, in several portions, after adding a few drops of toluene. It is then filtered off in the cold. Again mix the precipitate with dilute phosphate buffer, grind and filter. Now suspend the precipitate in about 100 ml. of 0.5 *M* phosphate buffer, pH 7.0. Add a few drops of toluene and grind again in the blender to suspend the precipitate. Keep in a bottle in the ice chest. This material will keep for 6 months, or longer. This suspension contains diaphorase, cytochrome oxidase, traces of the cytochromes a, b and c, and traces of various dehydrogenases.

227. Cytochrome Oxidase Preparation. This enzyme can be prepared from beef heart or from the hearts of other species. The method of preparation is identical with that described for diaphorase in Exp. 226. Diaphorase prepared in this manner contains, in addition to cytochrome oxidase, traces of cytochromes a, b and c, as well as traces of dehydrogenases.

228. Warburg Manometric Apparatus.* Reactions which occur in tissues, in tissue suspensions, or in enzyme preparations are frequently studied by following the changes which are produced in the amount of gas in equilibrium with the liquid containing the preparation being studied. The change in amount of gas results in a

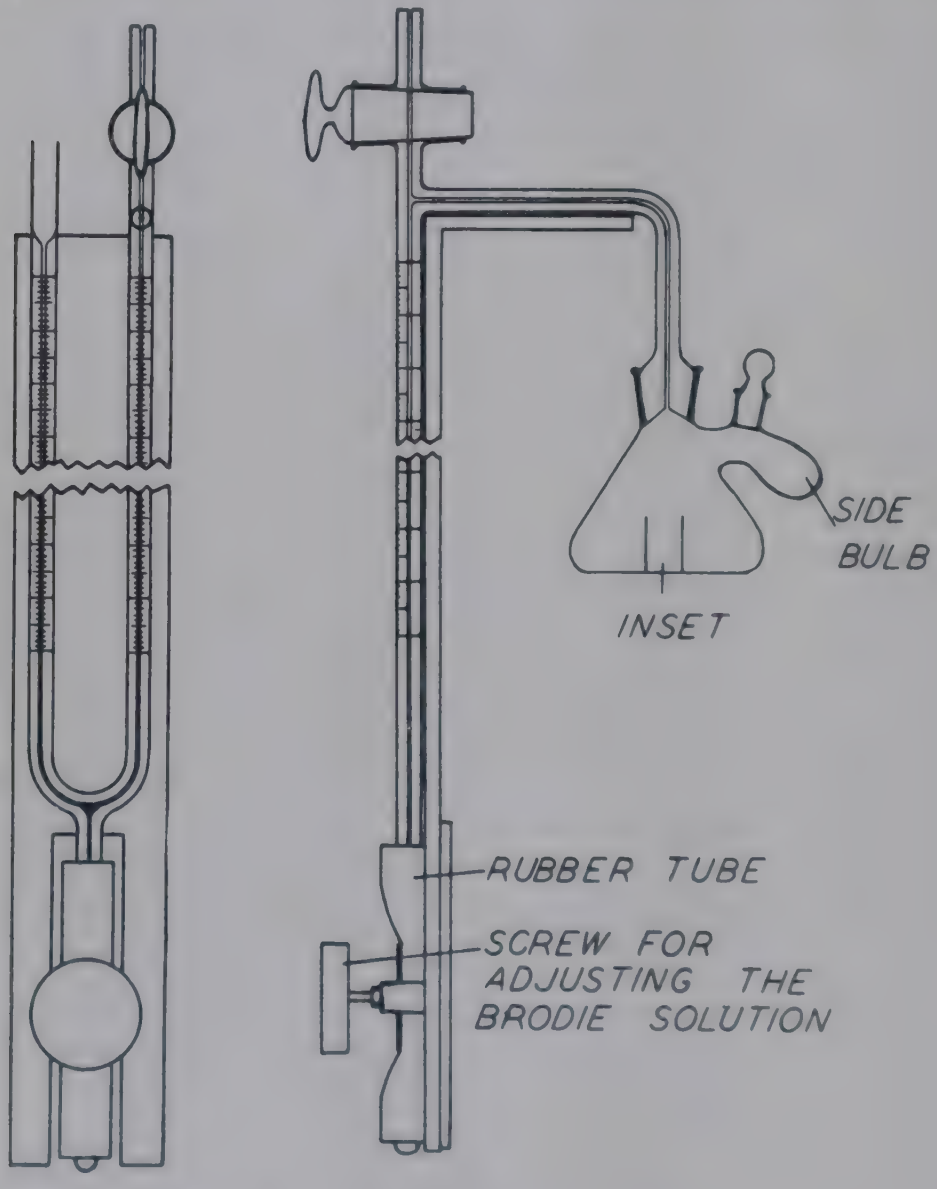


FIG. 18. Warburg manometer.

change in pressure or volume, or both, in the reaction vessel and this change in pressure and, or, volume can be followed readily by manometric methods. In recent years widespread application has been made of micro- or semi micro-manometric methods.

* This experiment is given as a demonstration or else is to be performed only under the direct supervision of the instructor.

Three kinds of changes are commonly studied by the use of manometric methods. These are, (1) carbon dioxide evolution or uptake, (2) oxygen evolution or uptake, and (3) changes in hydrogen ion activity of the solution. Changes in hydrogen ion activity are followed indirectly by using carbon dioxide gas in equilibrium with bicarbonate buffer solutions. As the pH changes, the partial pressure of the carbon dioxide changes. Hence any reaction which causes a change in pH can be followed.

Various types of manometric apparatus have been employed. Two general types have been widely used. One of these is the constant volume type of manometric apparatus shown in Fig. 18. This is commonly known as the Warburg manometer because of the very considerable application O. Warburg has made of this form of apparatus. The second type which has been widely used is commonly known as the differential, or Barcroft, manometer. In this form of the apparatus two flasks are used with each manometer and the manometer measures the difference in pressure in the two flasks. Both pressure and volume change during the reaction. The same fundamental principles are involved in the use of both of these types of manometric apparatus. The only differences are in the details of operation and the method of calculating the so-called vessel constant. Recently other forms of manometric apparatus have come into rather widespread use. (For a brief description see Perkins, J. J., *Ind. Eng. Chem., Anal. Ed.* **15**, 61 [1943].)

You can use your preparations of cytochrome C and cytochrome oxidase in this experiment. Prepare five flasks by adding the materials indicated in the following table:

Addition	Flask no.				
	1	2	3	4	5
	ml.	ml.	ml.	ml.	ml.
Water	2.7	0.5†	0.2†	—	0.2†
Buffer + cytochrome C	—	2.0	2.0	2.0	—
Cytochrome oxidase (in the side bulb)	—	0.2	0.2	0.2	0.2
Hydroquinone	—	—	0.3	0.3	0.3
20% KOH (in inset)	—	—	—	0.2	—
Buffer + cytochrome C + NaN_3	—	—	—	—	2.0

† 0.2 ml. in the inset.

The hydroquinone solution is prepared by dissolving enough hydroquinone in distilled water to give an $M/6$ solution. What is the concentration of hydroquinone in the final mixture in the flask? The buffer is $M/10$ phosphate buffer, pH 7.2; 10 ml. of this buffer is mixed with 2 ml. of your cytochrome C preparation. The solution containing sodium azide, NaN_3, is prepared using $M/10$ phosphate buffer, pH 7.2, which is $M/666$ with respect to NaN_3; 5 ml. of this buffer are mixed with 1 ml. of your cytochrome C preparation. The cytochrome oxidase which you have prepared previously is diluted with nine volumes of the $M/10$ phosphate buffer, pH 7.2 (without azide) before being used. One ml. of the undiluted oxidase preparation is placed in a weighing dish (or covered crucible) which has been dried at 100° C. and weighed. This aliquot is dried at 100° C. until it has a constant weight. Also dry an aliquot of the buffer in which the enzyme preparation is suspended. The difference in weight between the buffer and the buffer plus the enzyme is the dry weight of the enzyme. This will be used to calculate Q_{O_2}.

When the flasks are ready and the side plugs are snugly in place, carefully attach the flasks to the manometers. Each flask should be fitted to the manometer having the same number. The ground glass surface of the manometer connection should be smeared lightly with stopcock grease before the flasks are attached. Turn the flasks gently back and forth to seat them snugly onto the manometers. Fasten them in place with the small metal springs.

Be sure the stopcock at the top of the manometer is open and place the manometer on the holder of the shaker. The flasks should be immersed in the water of the bath at least up to the base of the manometer connection. The temperature of the bath should be about 38° C. Turn on the shaker and shake the flasks and manometers at about 100 to 120 strokes per minute for about 10 minutes. Then stop the shaker and turn the knob at the base of the manometer until the manometer fluid (Brodie solution) stands at the 150 mm. mark in the arm of the manometer connected to the flask. (The liquid should then be at the 150 mm. mark in the other arm also.) Close the stopcock at the top of the manometer and read the level of the fluid in the open arm of the manometer. Place your index finger over the open end of the manometer. Now lift the manometer from its mount and mix the contents of the side bulb with the contents of the main portion of the flask. Avoid spilling the KOH from the center inset. Note the time. Place the manometer back on its mount and again start the shaker. At intervals, *e.g.*, at 30 second intervals, the other flasks are

similarly mixed. Each manometer is read at 5 minute intervals after it has been mixed. To read the manometer, stop the shaker and adjust the manometer fluid to the 150 mm. mark in the closed arm of the manometer. Read the level of the liquid in the other arm. The readings should be made as rapidly as possible. After 30 minutes the readings may be discontinued. Record the results in tabular form.

The flask which contained only water should also be read every five minutes. This records changes due to temperature and pressure and is called a thermobarometer. The difference between the readings of the thermobarometer and the other flasks measures the amount of reaction which has occurred in the experimental flasks. These differences are used to calculate the results.

When the experiment is finished, open the stopcock at the top of the manometer and remove the manometers and flasks from the water bath. Remove the flasks and clean them. Be extremely careful, since the flasks are very expensive.

Calculation of results. All volume changes are expressed in mm^3, all readings of the manometers in mm., and all quantities of gas in mm^3 of dry gas under standard conditions, *i.e.*, 0° C. and 760 mm. Hg pressure.

Let

x = the change in volume of gas in the flask.

h = the corresponding reading of the manometer, *i.e.*, the difference between the reading for the experimental manometer and the thermobarometer. When a gas is being absorbed, h will be negative.

V_G = the volume of gas space in the vessel. This must include the volume of the connecting and manometer tubes to the 150 mm. mark.

V_F = the total volume of fluid in the vessel.

T = the temperature (absolute) of water bath.

P = the initial pressure in the vessel. This is usually equal to the barometric pressure plus the vapor pressure of the solution in the flask.

P_0 = the normal pressure (760 mm Hg). This is expressed in mm. of the manometer fluid. If D is the density of the fluid

$$P_0 = 760 \frac{13.60}{D}$$

p = the vapor pressure of water at temperature T.

α = the solubility of the gas which is absorbed in (or evolved

from) the liquid in the flask. This is commonly expressed in mm^3 of gas, under standard conditions, dissolved in 1 mm^3 of the liquid when in equilibrium with the gas at the partial pressure of P_0.

The initial volume of gas in the gas space, under standard conditions

$$= V_G \frac{273}{T} \frac{P - p}{P_0}$$

The initial volume of gas dissolved in the liquid in the vessel, under standard conditions

$$= V_{F\alpha} \frac{P - p}{P_0}$$

The final volume of gas in the gas space, under standard conditions

$$= V_G \frac{273}{T} \frac{P - p + h}{P_0}$$

The final volume of gas dissolved in the liquid in the flask

$$= V_{F\alpha} \frac{P - p + h}{P_0}$$

Obviously the change in volume of the gas in the vessel is equal to the difference in the volume of gas present initially and the volume present finally, which is

$$x = V_G \frac{273}{T} \frac{P - p + h}{P_0} - V_G \frac{273}{T} \frac{P - p}{P_0} + V_{F\alpha} \frac{P - p + h}{P_0} - V_{F\alpha} \frac{P - p}{P_0}$$

Combining the terms and assuming that there is no change in barometric pressure or temperature during the experiment, *i.e.*, that the initial and final values of P and p are equal

$$x = h\left(\frac{V_G \frac{273}{T} + V_{F\alpha}}{P_0}\right)$$

The quantity in parenthesis is a constant which can be evaluated by measuring the volume of the flask and connecting manometer tube to the 150 mm. mark. Hence

$$x = hk$$

The quantity k is the so-called flask, or vessel, constant. A number of points should be remembered concerning this quantity:

1. It is a function of the temperature of the water bath.

2. It depends upon the amount of liquid in the flask.

3. It depends upon the nature of the gas being evolved, or absorbed, since α is characteristic for each gas.

4. It depends to some extent upon the nature of the solution in the flask, since α is influenced slightly by the substances in solution. This is negligible with most gases except carbon dioxide.

5. The derivation of this constant assumes that there is no change in the barometric pressure or temperature during the experiment. This is not always true. However, changes in barometric pressure and temperature are corrected for by use of the thermobarometer when changes in the latter are used to correct the observed values of h.

You will be given the volumes of the flasks and the connecting manometer tubes to the 150 mm. mark. These were obtained by filling the flasks with mercury and attaching them to the manometer. The manometer is then inverted and filled with mercury to the 150 mm. mark. Then all of the mercury is removed from the flask and manometer, its temperature is measured, and it is weighed. From the result one calculates the volume of flask and its manometer to the 150 mm. mark. The density of Brodie solution is about 1.03. In the following table values are given for α for water at a partial pressure of 760 mm. Hg.

Gas	*30°*	*38°*	*40°*
Carbon dioxide	0.66	0.55	0.53
Nitrogen	0.014	0.0122	0.012
Oxygen	0.026	0.024	0.023

Use the value of α given in this table and calculate k. Calculate x for each reading with each manometer and plot the results. Was appreciable carbon dioxide evolved in this experiment? *Caution:* Sometimes KOH is spilled into the main portion of the vessel and as a result the cytochrome oxidase has little or no action due to the resulting change in pH. Why was hydroquinone added? What was the effect of NaN_3? Calculate Q_{O_2}. Q_{O_2} is the mm.^{3} of oxygen, at standard conditions, absorbed per hour per mg. dry weight of enzyme preparation. If the curve for oxygen absorption is not a straight line, use the initial rate, *i.e.*, the initial slope of the curve to calculate Q_{O_2}.

Hydroquinone is not a very satisfactory substrate for this experiment since it is oxidized to benzoquinone, which slows down the action of the enzyme. The effect of the quinone can be avoided somewhat by using semicarbazide. A more satisfactory substrate for use in measuring cytochrome oxidase activity is ascorbic acid (Schneider, W. C., and Potter, V. R., *J. Biol. Chem.* **149**, 217 [1943]), but this requires that all of the reagents, preparations, and water be free of copper contamination.

Copper ions catalyze the oxidation of ascorbic acid and hence would introduce an appreciable error in this experiment.

Brodie solution for use in the manometers contains the following ingredients:

Sodium chloride	23 g.
Sodium taurocholate	5 g.
Water	500 ml.

A few drops of a strong alcoholic solution of thymol (enough to produce a noticeable smell of thymol) and a few crystals of Congo red are added to the solution.

The Warburg apparatus cannot be used with solutions containing volatile liquids, such as ether. Even hydrocyanic acid will interfere.

Additional information concerning manometric methods will be found in the following:

Dixon, M., Manometric Methods, Macmillan, 2d ed. (1943).

Warburg, O., The Metabolism of Tumors, Translated from the German by Dickens, F., Constable and Co. (1930).

Krebs, H. A., in Oppenheimer, C., Die Fermente und ihre Wirkungen, Georg Thieme, pp. 635–670 (1929).

Dickens, F., in Bamann and Myrbäck, Die Methoden der Fermentforschung, Georg Thieme, pp. 2455–2455 (1941).

Steiner, M., in Bamann and Myrbäck, Die Methoden der Fermentforschung, Georg Thieme, pp. 2682–2690 (1941).

Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Burgess (1945).

229. Determination of Amylase Activity by Method of Willstätter, et al. (Willstätter, R., Waldschmidt-Leitz, E., and Hesse, A. R. F., *Z. physiol. Chem.* **126**, 143 [1923]).

Place in a 50 ml. glass-stoppered bottle 25 ml. of freshly boiled Kahlbaum's soluble starch (1 per cent), 10 ml. of 0.2 *M* phosphate buffer (5.1 ml. 0.2 *M* KH_2PO_4 and 4.9 ml. 0.2 *M* $Na_2HPO_4 \cdot 2\,H_2O$), and 1 ml. of 0.2 *M* sodium chloride, Kahlbaum. Mix and bring to 37° C. in a water bath. Add 1 ml. of enzyme solution and mix at once. After just 10 minutes, add 2 ml. of *N* hydrochloric acid to stop enzyme action. Wash with a little water into an Erlenmeyer flask. Pipette into the flask 20 ml. of 0.1 *N* iodine solution, then add 60 ml. of 0.1 *N* sodium hydroxide and mix. After 10 minutes add 10 ml. of 10 per cent sulfuric acid and titrate the excess iodine with 0.1 *N* thiosulfate. Now run a blank determination.

Calculation of Amylase Units. The ml. of 0.1 *N* iodine used up by the blank are subtracted from the ml. of iodine used by the determination and the result is multiplied by the factor 17.15. This gives the mg. of maltose formed. This figure is then employed to figure out the monomolecular reaction constant where the complete hydrolysis of the starch would produce 0.1875 g. of maltose. The reaction constant represents the amylase units. An example of the calculations is given below:

A solution of pancreas glycerine-extract was diluted 250 times and 0.8 ml. taken for the determination. The titration showed that 2.29 ml. of iodine were used up. The blank showed that 0.53 ml. of iodine were used up. The difference was 1.76 ml. of 0.1 *N* iodine. This multiplied by 17.15 gave 30.2 mg. of maltose formed. Using the monomolecular equation:

$$K = \frac{1}{t} \log_{10} \frac{\text{total maltose}}{\text{total maltose} - \text{maltose formed in 10 minutes}}$$

we have:

$$K = \frac{1}{t} \log_{10} \frac{0.1875}{0.1875 - 0.0302}$$

this figures out to be $K = 0.0076$. Hence 0.8 ml. of diluted extract contained 0.0076 amylase units.

Our glycerol pancreas extracts when undiluted contain about 3.12 units per ml. Pancreas powder contains about 1 unit per mg. It is necessary to employ diluted amylase for this determination, and the amount of maltose formed should not exceed 100 mg. About 0.6 ml. of the 0.1 *N* iodine should be employed for every mg. of maltose formed. The volume of 0.1 *N* sodium hydroxide added should amount to 1.5 times the volume of iodine added, in addition to the amounts required to neutralize the hydrochloric acid and to convert the primary potassium phosphate into secondary potassium phosphate.

230. Preparation of Almond Meal.

Five students are asked to work together.

Shell about 2 pounds of raw almonds and soak several hours in luke warm water. Remove the skins. Allow the almond meats to dry over night on a clean towel. Now grind the meats in a meat chopper and place the material in a large-mouth glass bottle under petroleum ether. (Danger of fire.) Filter off the material in the hood and again place under petroleum ether. Next day filter off the material and wash with petroleum ether. Dry in a flame-free room on filter paper. The next day grind in a ball mill for 8–11 hours. Place

the ground material under petroleum ether for several hours and then filter off. Again extract with petroleum ether. Dry on filter paper and place in a bottle.

231. Preparation of β-Glucosidase from Almond Meal (Sumner and Howell).

Two students are asked to work together.

Stir 10 g. almond meal with 100 ml. of one-half saturated ammonium sulfate (37.7 g. per 100 ml. water) for 5 minutes. Filter. Discard the filtrate and extract the residue with 100 ml. of one-quarter saturated ammonium sulfate and filter. Add to each 10 ml. of filtrate 2 g. of solid ammonium sulfate. Filter off the precipitate of β-glucosidase. Mix with a little water and dialyze in the ice chest to remove the ammonium sulfate and to precipitate inactive globulin. Centrifuge off the precipitated globulin. Decant the enzyme solution. Add toluene and keep in the ice chest.

232. Determination of β-Glucosidase Activity (Method of Sumner and Howell).

Place 5 ml. of salicin solution in a small test tube and allow to come to 30° C. in a thermostat bath. Now add 1 ml. of β-glucosidase solution. Mix at once, noting the time. After exactly 20 minutes add 5 ml. of 0.1 *N* sodium hydroxide and mix at once.

Pipette 1 ml. of this solution into a Folin-Wu sugar tube and add 3 ml. of the dinitrosalicylic sugar reagent and mix. Heat the tube for 5 minutes in boiling water. Cool, dilute to 25 ml. and mix. Read in the photoelectric colorimeter. Calculate the mg. of glucose formed by the enzyme in the total 11 ml. of digest (see Exp. 50).

Calculate the units of β-glucosidase from the amount of glucose formed. Since the amount of glucose formed is not directly proportional to amount of enzyme present a curve must be used. A curve which shows the relationship between the amount of enzyme and the amount of glucose formed in 20 minutes at 30° is given in Fig. 19. This curve was obtained by allowing 0.1, 0.2, 0.4, 0.6, and 1.0 ml. respectively, of an enzyme solution to act upon salicin under the standard conditions. A unit of β-glucosidase will hydrolyze 50 per cent of the salicin in the digest in 20 minutes at 30° C. This corresponds to a production of 9 mg. of glucose. It will be noticed that it required 0.36 ml. of the enzyme solution used to construct the curve in Fig. 19 to produce this amount of glucose. Consequently, 0.36 ml. of this enzyme solution contains one β-glucosidase unit.

The number of β-glucosidase units in an unknown enzyme solution may be calculated as follows: Suppose 1 ml. of an unknown enzyme

solution formed 5 mg. of glucose under the prescribed conditions. The curve in Fig. 19 shows that 5 mg. of glucose corresponds to 0.16

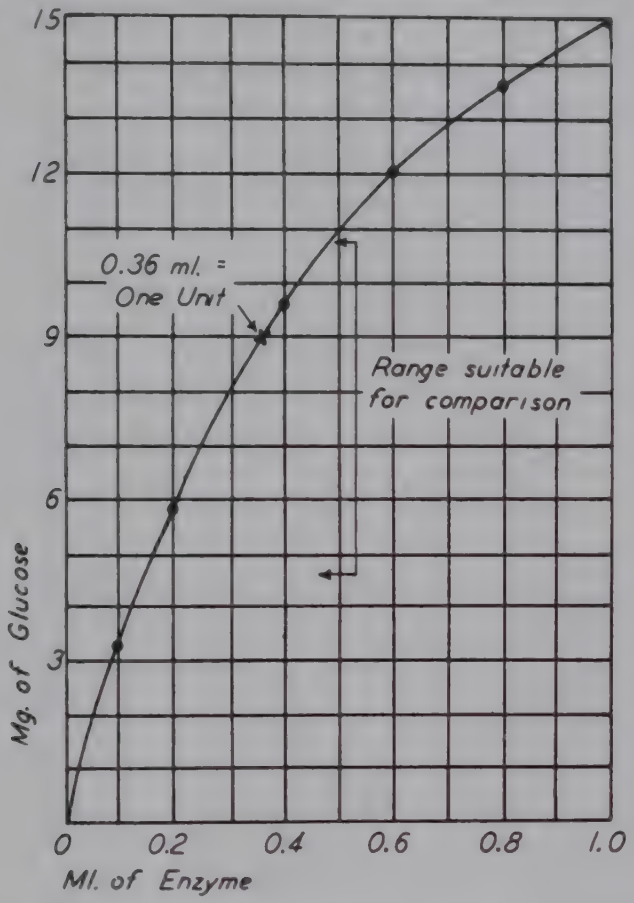


FIG. 19. β-Glucosidase activity curve.

ml. of the enzyme solution used to construct the curve. Since one unit of β-glucosidase corresponds to 0.36 ml. of this latter enzyme, the unknown enzyme solution contains:

$$\frac{0.16}{0.36} = 0.44 \text{ Units of } \beta\text{-Glucosidase per ml.}$$

Salicin Solution. Weigh out 5.72 g. of salicin (Kahlbaum) and make it to 1000 ml. volume by dissolving in acetate buffer of pH 4.4 and mix well. The acetate buffer can be prepared by adding 630 ml. of 0.1 *N* acetic acid to 370 ml. of 0.1 *N* sodium acetate. If protected from the light the salicin solution will last several months.

233. Determinations of Lipase Activity by Method of Willstätter (Willstätter, R., Waldschmidt-Leitz, E., and Memmen, F., *Z. physiol. Chem.* **125**, 93 [1923]).

Dilute 1 ml. of glycerol pancreas extract (1–3) to 30 ml. and place 10 ml. of this in a 25 ml. glass stoppered bottle. Now rapidly add 2.5 g. of olive oil, 2 ml. of ammonia-ammonium chloride buffer, and

0.5 ml. of 2 per cent calcium chloride solution. Shake for a moment. Note the time. Now add 0.5 ml. of 3 per cent albumin solution and shake violently for 3 minutes. Place the flask in a thermostat bath at 30° C. Sixty minutes after adding the olive oil, wash the digest into an Erlenmeyer flask, using enough neutral 95 per cent alcohol to make a total volume of about 125 ml. Add 20 ml. of ether at once and then 12 drops of 1 per cent thymolphthalein. Titrate to a deep blue with 0.2 *N* alcoholic sodium hydroxide.

Now run a blank with the same reagents and subtract this from your titration. Assuming the olive oil to be triolein, calculate how much of it has been hydrolyzed. One lipase unit will hydrolyze 24 per cent of the oil under the above conditions. The lipase value is the number of units in 10 mg. of dry enzyme. With 24 per cent hydrolysis, the pH of the digest has become 5.5. Upon further hydrolysis the rate of enzyme action decreases greatly.

The authors of this method found in one experiment that 40 mg. of pancreas powder required 14.0 ml. of 0.2 *N* alkali for titration and that the blank required 7.05 ml. The difference, or 6.95 ml., corresponded to a hydrolysis of 16.4 per cent of the oil.

The olive oil should have a saponification value of 185.5.

The buffer is made by adding 66 ml. of *N* ammonium hydroxide to 134 ml. of *N* ammonium chloride. This solution has a pH of 9.2 at 18° C. and 8.9 at 30° C.

234. The Determination of Peroxidase Activity (Willstätter, R.,* and Stoll, A., "Untersuchungen über Enzyme," vol. I, p. 414 [1928]).

This method has been modified only in that one-tenth quantities of reagents are employed.

Place in a 250 ml. flask 190 ml. of redistilled water and 10 ml. of purest, freshly-prepared 5 per cent pyrogallol solution. Add 1 ml. of 0.5 per cent hydrogen peroxide (prepared by diluting Merck's 30 per cent perhydrol). Allow the solution to come to 20° C. in the thermostat bath and then add 1 ml. of peroxidase solution of the proper dilution. Mix at once and note the time of addition. After 5 minutes add 5 ml. of 10 per cent sulfuric acid and mix at once. Now add about 25 ml. of ether and shake to extract the purpurogallin. Separate in a separatory funnel, running the ethereal solution of purpurogallin into a 100 ml. graduate. Continue the extraction with smaller quantities of ether until the yellow ethereal extract amounts to just 50 ml. There will be some water in the bottom of the 100 ml. graduate and this must be allowed for in estimating the volume of the extract.

* See also Sumner, J. B. and Gjessing, E. C., *Arch. Biochem.* 2, 291 [1943].

Now, mix the ethereal extract well, filter, and read in the photoelectric colorimeter, using a blue glass filter. Find out from the standard graph how much purpurogallin you have in the unknown ethereal solution. If the amount is more than 5 mg. or less than 2 mg., the analysis will have to be repeated, using less or more peroxidase, as the case may be.

Willstätter's peroxidase unit is that amount of enzyme that will form 1000 mg. of purpurogallin under the conditions described above. Calculate what fraction of a unit of peroxidase is present in one ml. of your undiluted peroxidase solution.

If the dry weight of enzyme in one ml. is known it will be possible to calculate also the *P.Z.*, or Purpurogallin Zahl. This is simply the mg. of purpurogallin formed under the above mentioned conditions per mg. of dried enzyme. Thus, if one mg. of peroxidase should form 1000 mg. of purpurogallin it would have a *P.Z.* of 1000. Theorell has found his crystalline horse radish peroxidase to have a *P. Z.* of about 900.

The purpurogallin standard can be prepared by dissolving exactly 100 mg. of pure, dry purpurogallin in enough ether to bring the volume up to 1000 ml.

Purpurogallin can be prepared as follows: Place one liter of 6 per cent pyrogallol and 10 ml. of a powerful peroxidase solution in a 2 liter flask and arrange to stir with a glass stirrer attached to a small electric motor. Now add from a separatory funnel 530 ml. of 3 per cent hydrogen peroxide. The peroxide solution must be added very gradually, allowing about 3 hours time for all to drip into the reacting mixture. When all of the peroxide has been added, filter off the crystals of purpurogallin upon a hardened filter paper, using suction. Wash well with distilled water. Dissolve in boiling 95 per cent alcohol. Filter and add 6 volumes of water to the filtrate. Now filter off the precipitated purpurogallin. Again dissolve in boiling alcohol, filter, and precipitate with 6 volumes of water. Filter by suction and dry in a desiccator.

The standard graph is prepared by making readings of ether solutions containing 10, 8, 6, 4, and 2 mg. of purpurogallin per 100 ml. of ether and then plotting these readings against mg. of purpurogallin. The photoelectric colorimeter is first set at the null point (or, at 100, depending on the colorimeter used) using pure ether in the colorimeter tube.

235. Determination of Catalase Capability (Euler, H., von, and Josephson, K., *Ann. Chem.* **452**, 158 [1927]).

Pipette 50 ml. of 0.005 to 0.015 *N* hydrogen peroxide (made up to

contain $M/150$ phosphate buffer of pH 6.8) into a 150 ml. Erlenmeyer flask and bring to 0 to 1° C. in a thermostat bath of ice and water. Add 1 ml. of catalase solution of proper dilution, mix at once, and at once remove a 5 ml. sample and pipette at 0 time into an Erlenmeyer flask which contains 15 ml. of 2 N sulfuric acid. Mix at once. Now titrate to the first faint pink color by running in 0.005 N permanganate from a burette.

After 3 minutes, remove a second 5 ml. sample and titrate as before. After 6 minutes remove and titrate a third 5 ml. sample. Repeat after 9 and 12 minutes. The times of removal of samples need not be 3, 6, 9, and 12 minutes, but can be any other reasonable number of minutes provided the times are taken with accuracy.

Now calculate the velocity constants, using the equation:

$$K = \frac{1}{T} \log_{10} \frac{A}{A - X}$$

Here the velocity constant is K; the minutes of digestion are T; A is the titration value of 5 ml. of the digest at 0 time in terms of 0.005 N permanganate; and $A - X$ is the titration value after T minutes. Plot the various values obtained for K against the time and extrapolate to estimate the value of K at 0 time. The value found for K should be in the neighborhood of 0.02 to 0.05, and if much greater or much smaller it will be necessary to employ a greater or else a smaller dilution of the catalase, as the case may be.

In order to calculate the activity of the catalase preparation, or the "*Katalasefähigkeit*," one employs the equation:

$$\textit{Katalasefähigkeit} = \frac{\text{velocity constant}}{\text{g. enzyme preparation added to the 50 ml. of peroxide}}$$

The "*Kat. f.*" of a crude liver extract may be approximately 300 while the "*Kat. f.*" of crystalline beef liver catalase is 30,000.

Method of Jolles (Jolles, A., *Münch. med. Wochschr.* **51**, 2803 [1904] quoted from von Euler, Chemie der Enzyme, Teil II, p. 73). Instead of employing potassium permanganate, one can determine the undecomposed hydrogen peroxide in the acidified aliquots of the digest by adding 10 ml. of 10 per cent potassium iodide and 1 drop of 1 per cent ammonium molybdate and mixing. The iodine set free is titrated 3 minutes later with 0.005 N thiosulfate, using starch towards the end of the titration. The values of thiosulfate are employed to calculate the monomolecular constants.

Determination of Dry Weight of Enzyme Preparation. The deter-

mination of grams of catalase per ml. of solution can be accomplished by pipetting 1 ml. or more of the catalase solution into a weighed platinum crucible and evaporating to dryness in an oven at 95° C. The crucible is then cooled for an hour in a desiccator containing phosphorus pentoxide and weighed. After the weighing, the crucible is again heated in the oven, cooled in the desiccator, and weighed; and this process is repeated until the weight is constant. It must be kept in mind that the enzyme preparation may be deliquescent.

The ash content of the enzyme is obtained by ashing with an oxidizing Bunsen flame at low red heat, cooling in the desiccator, and weighing. With some enzyme preparations it is often impossible to say whether the ash found is an integral part of the enzyme or an impurity. If ammonium salts are present in the enzyme preparation, they will be volatilized entirely and will not be present in the ash. At any rate, it is always best to determine the dry weight of any enzyme solution only after this has been dialyzed until salt-free. It must be admitted, however, that some enzymes will not suffer dialysis without loss of activity. In this event it probably would be unobjectionable to dialyze against some such salt as sodium sulfate and to analyze the ash later for sulfate and subtract.

236. Estimation of Lipoxidase (Sumner, J. B., and Smith, G. N., *Arch. Biochem.* **14**, 87 [1947]).

Place in a clean, dry test tube (18 × 150 mm.) 0.1 ml. of neutralized fatty acid solution. Add 0.5 ml of the stock bixin solution. Mix well and add 5 ml. of gum arabic-phosphate. Place the tube in a thermostat bath at 25° C. After the contents of the tube have had time to reach the temperature of the bath add from 0.1 to 1.0 ml. of properly diluted lipoxidase solution; mix gently, starting a stop watch at the same time. By visual comparison determine the time required for the test to become decolorized until it has the same color as a standard tube. This tube is prepared by mixing 0.1 ml. of the fatty acid solution, 0.1 ml. of the stock bixin solution, 5 ml. of the gum arabic-phosphate and enough water to bring this standard to the same volume as that of the unknown.

A lipoxidase unit is defined as that quantity of enzyme which will bleach the unknown to the same intensity as the standard within 40 seconds at 25° C. and at pH 6.5.

Fatty Acid Solution. The fat of freshly ground soybeans is extracted with petroleum ether and the ether is then distilled off. The fat is hydrolyzed with sodium hydroxide. The fatty acids are set free by addition of excess 10 per cent sulfuric acid, are filtered off and washed well with distilled water. One dissolves 1.0 ml. of the melted fatty acids in 100

ml. of 95 per cent alcohol and neutralizes to phenolphthalein with 0.1 *N* sodium hydroxide. The solution is diluted with 95 per cent ethyl alcohol to a volume of 300 ml.

Bixin Solution. Dissolve crystalline bixin in enough 95 per cent alcohol to give a concentration of 0.02 mg. per ml. If necessary a few drops of 0.1 *N* sodium hydroxide can be added to assist in dissolving the bixin, but the final solution must not be alkaline.

Gum Arabic-Phosphate. Add 40 g. of powdered gum arabic to 500 ml. of distilled water and heat on a steam bath until dissolved. Add 200 ml. of 0.5 *M* phosphate buffer of pH 6.5. Heat for 1 or 2 hours more on the steam bath. Filter and preserve with toluene. The solution may require a second filtration after several days.

Lipoxidase Solution. Stir 100 g. of finely ground defatted soybean meal with 500 ml. of ice cold water in the cold room. Add with stirring 10 ml. of 2 *N* acetic acid. Filter rapidly in the cold room, allowing the filtrate to drip into about 50 ml. of 0.5 *N* disodium phosphate. Stir this to mix.

237. Estimation of Glutamic Acid, Using Glutamic Acid Decarboxylase. (Schales, O., and Schales, S., *Arch. Biochem.* **11**, 445 [1946]). Grind 90 g. of fresh squash in a Waring blender with 300 ml. of water for 4 minutes. Allow to stand in the ice chest for about 1 hour. Centrifuge at high speed. The nearly clean supernatant liquid is decanted. It contains the enzyme. This solution can be frozen and dried in the frozen state. The dried material can be kept at room temperature over fuzed calcium chloride without loss of activity. The dry powder from 90 g. of squash can be dissolved in 30 ml. of phosphate buffer of pH 5.75 and *M*/15.

Estimation of Enzyme Activity. Employ the Warburg apparatus at 37° C., using vessels with two side bulbs. The main vessel contains 4 ml. of enzyme solution, the first side vessel contains 0.5 ml. of a solution containing from 0.5 to 2.0 mg. of *L*(+) glutamic acid, and the pH is from 6 to 7. This solution must be free from carbonate. The second bulb contains 0.5 ml. of 1.2 *N* sulfuric acid. A blank vessel is prepared, using 0.5 ml. of water instead of glutamic acid.

After equilibration for 20 minutes, the contents of the first bulb are tipped into the main flask. Liberation of carbon dioxide is complete after 15 or 20 minutes, however, the reaction is allowed to continue for 1 hour. The contents of the second bulb are then tipped into the main flask and, after shaking for 10 minutes the reading is taken. Corrections are made for the blank. Pure glutamic acid produces an average of 102.6 per cent of the theoretical quantity of carbon dioxide.

ALCOHOLIC FERMENTATION

238. Preparation of Autolyzed Dried Yeast (for the preparation of Lebedew maceration juice) (Nilsson, R., in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, p. 1284 [1940]).

Bottom brewer's yeast is washed and pressed until its water content is decreased only enough to give a yeast of doughy consistency. Allow to stand overnight at room temperature. Grind through a meat grinder with a sieve plate with holes 4 mm. in diameter. Spread the ground material out in a layer about 2 cm. thick on filter paper. Allow to dry at 30° C. The drying should take from 2 to 3 days and the yeast should turn brown. Autolysis occurs. If the drying is too rapid the material can be placed for a time in a moist chamber. Grind the dry material in a coffee mill and store it in a bottle with a paraffined stopper in the cold room.

The autolyzed dried yeast usually consists entirely of dead cells which can not grow and divide.

239. Preparation of Yeast Dried Without Autolysis (for apozymase preparation) (Nilsson, *ibid.*).

Wash fresh bottom brewer's yeast several times by stirring with water and filtering off in a cold place. Do not allow to freeze. Wash until the washings are nearly colorless. Now place the yeast on a piece of canvas, wrap up and press out as much of the water as possible in a Buchner press. Next, sieve the yeast through a sieve with holes about 1 mm. in diameter, upon a filter paper. The layer of sieved yeast must not be more than 3 mm. thick. Dry rapidly. If it is desired to kill all the cells the drying must continue at 37° C. for 24 hours. Grind the dried yeast in a coffee mill and place the powder in a dark glass bottle. Store it in the cold room.

When the yeast cells are dead they take up methylene blue from a solution of phosphate buffer and methylene blue. A much better test is to add the yeast to a sterile nutrient agar medium. This is poured into sterile Petri dishes. The covers are put on and the tests are incubated at 30°. If no yeast colonies appear, all of the cells were dead.

240. Preparation of Yeast Press Juice (Buchner) (Nilsson, *ibid.*).

Yeast is washed and is then pressed for 5 minutes in a Buchner press at 50 atmospheres pressure. The yeast is then mixed with its weight of quartz sand and one-half of its weight of infusorial earth. The mixture is ground in a mill until it has a pasty consistency. The ground mixture is allowed to stand 2 hours at room temperature.

It is then mixed with enough infusorial earth to become powdery and is wrapped in a canvas cloth and pressed in a Buchner press at about 300 atmospheres pressure. The juice is collected and is centrifuged clear. This is "zymase." It will bring about a cell-free fermentation of glucose.

This juice does not keep very well, even at low temperatures. A stable, dry preparation may be obtained from the juice by the method of Buchner and Hahn (see Nilsson, *ibid.*): 50 ml. of yeast press juice is poured, with vigorous stirring, into a mixture consisting of 400 ml. of absolute ethyl alcohol and 200 ml. of ether. The precipitate is filtered off rapidly with suction and is washed, first with absolute alcohol, and then with ether. The material is then dried in a vacuum desiccator over sulfuric acid. "The entire preparation takes about 6 minutes."

241. Preparation of Lebedew Maceration Juice (von Lebedew, A., *Z. physiol. Chem.*, **73**, 447 [1911]).

Place dried autolyzed bottom yeast in a thick-walled flask or bottle and mix with 3 times its weight of water. Keep at 35° C. for 2 hours, stirring every 15 minutes. Centrifuge and use the clear supernatant juice. This will ferment sugar. Mix 10 ml. of the juice with 10 ml. of sucrose-phosphate and test for fermentation in a fermentation tube. With maceration juice from some yeasts it may be necessary to add also a few drops of 10 per cent $MgCl_2$ solution to obtain rapid fermentation.

The sucrose-phosphate solution is prepared by dissolving 50 g. of sucrose, 14 g. of KH_2PO_4 and 32 g. of NaH_2PO_4 in 500 ml. of water.

This maceration juice does not keep well. A stable, dry powder can be obtained by precipitation with alcohol, alcohol-ether mixture or acetone.

242. Preparation of Apozymase (Nilsson, *ibid.*).

Shake 25 g. of quickly-dried bottom brewer's yeast for one-half hour with 1500 ml. of water, then centrifuge down the yeast. Repeat twice, shaking only a few minutes. The resulting material should be free from cozymase. It is sometimes low in magnesium. This apozymase may be used directly or it can be dried by spreading upon clean biscuitware. After 15 minutes it is scraped off and is dried fully on filter paper.

243. Preparation of Aetiozymase.

Auhagen (see Nilsson, *ibid.*) prepares aetiozymase by shaking 2 g. of dry, non-autolyzed, bottom yeast with 250 ml. of water for 15 minutes and centrifuging. The material is next extracted with 100 ml. of 0.2 *M*

phosphate buffer of pH 7.8 for 30 minutes. . This is repeated once. Then the material is extracted twice with water to remove the phosphate.

Aetiozymase contains no cozymase, cocarboxylase, or magnesium.

244. Preparation of Coenzyme 1 (Sumner, J. B., Krishnan, P. S., and Sisler, E. B., *Arch. Biochem.* **12**, 19 [1947]). Mix 1 lb. (454 g.) of baker's compressed yeast in a pail in the hood away from any flame with 350 ml. of diethyl ether. Add 22 g. (caution) of conc. H_2SO_4 to 350 ml. of 95% ethyl alcohol and cool to room temperature. Add this to the yeast and ether. Stir for about 10 minutes. Now centrifuge. Pour off and save the clear brown supernatant. Add to it 2 volumes of 95% ethyl alcohol and let cool overnight in the ice chest. Centrifuge off the precipitate of crude coenzyme 1. Wash this twice with absolute alcohol and then once with ether. Dry in a desiccator. Yield about 3 g.

Further purification can be achieved by dissolving about 25 g. of the crude coenzyme 1 in water and adsorbing it on its own weight of acid-washed Norite. The adsorption complex is washed twice with water, then twice with dilute (1 per cent) ammonia and then twice with water. The coenzyme is eluted by shaking for an hour with 100 ml. of water and 10 ml. of isoamyl alcohol. The Norite is centrifuged down and the coenzyme 1 solution is decanted off. It is then evaporated at about 40° C. by placing it in an evaporating dish on a steam bath and directing air from an electric fan towards the surface of the liquid. When the liquid has evaporated to a very small bulk it is mixed with twice its volume of absolute ethyl alcohol and centrifuged. The clear supernatant is mixed with 2 more volumes of alcohol, acidified with a drop of 7.5 *N* sulfuric acid, stirred, and the precipitated coenzyme 1 is centrifuged down. The supernatant liquid is discarded. The precipitate of coenzyme 1 is washed twice with absolute ethyl alcohol and once with ether and then dried in an evacuated desiccator.

245. Preparation of Fructose-1,6-diphosphate (Robison, R., and Macfarlane, M. G., in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, p. 298 [1940]).

50 g. of quickly-dried yeast, 180 ml. of water and 30 g. of sucrose are mixed in a two liter flask which is connected with a calibrated vessel, for adding the phosphate solution, and also with a eudiometer filled with saturated salt solution. The eudiometer is for measuring the carbon dioxide which is formed. The fermentation is carried out at 20° to 25° C. and its progress is followed by measuring the CO_2-evolution. The flask must be vigorously shaken before each measurement. The fermentation begins after an induction period of about

one hour. The rate of CO_2-evolution increases at first and then falls to a basal value as soon as the inorganic phosphate in the yeast has been esterified. A solution of KH_2PO_4 ($M/2$) and sucrose (M), saturated with CO_2, is added from time to time in quantities of 60 to 80 ml. The rate of fermentation increases after each addition and decreases again as soon as the added phosphate is used. According to the equation of Harden and Young:

$$2\,C_6H_{12}O_6 + 2\,H_3PO_4 = C_6H_{10}O_4(PO_4H_2)_2 + 2\,CO_2 + 2\,C_2H_5\cdot OH + 2\,H_2O$$

the total "extra" carbon dioxide, in excess of the basal value, is equivalent to the esterified phosphate. Phosphate must be added periodically, as soon as the fermentation rate falls almost to the basal value. The addition must be discontinued when the periods required between additions become too long. In general one can add 300 to 400 mg. of phosphate in 60 ml. portions at intervals of 25 to 45 minutes. At the end of the fermentation, enough trichloroacetic acid is added to give a concentration of 5 per cent. The precipitated protein is filtered off after one-half hour.

After adjusting to pH 8.0 with sodium hydroxide, the filtrate is treated with barium acetate. Use an amount of the latter which is equivalent to 10 per cent more than the total phosphorus in the filtrate. The precipitate, which contains barium phosphate and the sparingly soluble barium salt of hexose diphosphate, is filtered off, washed with water and then with alcohol and dried in a vacuum desiccator over sulfuric acid.

The crude barium hexosediphosphate can be purified by dissolving it in 5 parts of water and adding enough HCl to give a pH of 4.0. One-fourth volume of alcohol is added with vigorous stirring. The precipitate is filtered off, washed with absolute alcohol, dried *in vacuo*, redissolved in water, adjusted to pH 4.0 and again precipitated with alcohol. This process is repeated several times. For the preparation of the neutral barium salt, the acid is dissolved in 200 parts of water. The solution is adjusted to pH 8.0 with cold, filtered barium hydroxide solution and an equal volume of alcohol is added. The salt is filtered off, washed with alcohol and is dried in a vacuum desiccator.

The best criterion of purity of this ester is the rate of hydrolysis in 1 *N* HCl at 100° C. Inorganic-P and total-P determinations are also indicative.

Rate of Hydrolysis in 1 N HCl at 100° C.

Time *mins.*	P hydrolyzed off *per cent*
7	31.8
10	37.8
30	58.9
60	71.9
180	93.6

Hexosediphosphate is also characterized by the fact that it gives a test for fructose and by the fact that 80 per cent of the phosphate is hydrolyzed off in $N/5$ sodium hydroxide at 100° C. in 3 minutes.

246. Diphosphoglyceric Aldehyde Dehydrogenase (Sumner, J. B., and Krishnan, P. S.. *Enzymologia,* XII, 232 [1948]). Pipette 1 ml. of

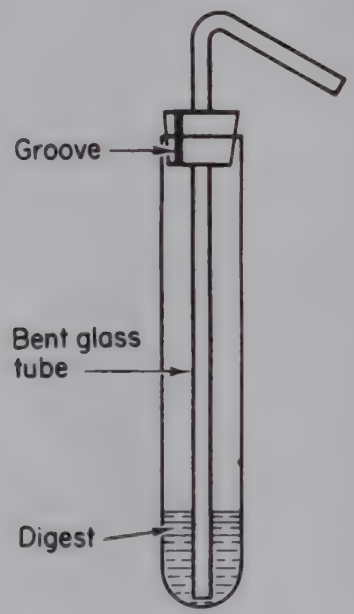


Fig. 20. Tube for observing the action of diphosphoglyceric aldehyde dehydrogenase.

apozymase suspension containing triosephosphates into a dry test tube (18 × 150 mm.). Pipette into the tube, using a serological pipette, 0.1 to 0.3 ml. of solution of coenzyme 1, containing 10 to 50 γ of coenzyme. Add 1 drop of caprylic alcohol and 0.2 ml. of 0.5 M phosphate buffer, pH 8.0. Seat in the test tube a bent glass tube passing through a cork stopper. The stopper has a groove cut in its side to allow gas to escape. The bent tube should reach to the bottom of the test tube as is shown in Fig. 20.

Place the tube in a thermostat bath at 25° C. and slowly pass oxygen-free nitrogen through the solution for about 10 minutes in order to remove all oxygen. Now add to the tube 0.1 ml. of 1–5000 methylene

blue and continue passing nitrogen through the solution. Note how long it is before the methylene blue is completely decolorized. It will require 2 to 4 minutes for complete decolorization when 8 γ of purified coenzyme 1 is present.

The oxygen-free nitrogen is prepared by passing nitrogen from a tank of the compressed gas through four 6-liter bottles containing 5 per cent sodium sulfite, 5 per cent pyrogallol and 5 per cent sodium hydroxide. The nitrogen is next bubbled through neutral potassium phosphate solution to remove alkaline spray. In preparing the alkaline pyrogallol-sulfite solution it is best to add the sodium hydroxide only after most of the oxygen has been removed by a stream of nitrogen.

The brewer's bottom yeast is prepared as in Exp. 239. To prepare apozymase one stirs 10 g. of the dried yeast with 500 ml. of distilled water and 5 ml. of 0.5 *M* phosphate buffer of pH 8.0 for an hour, using an electric stirrer. The yeast is then centrifuged down and the washing is discarded. The yeast is stirred twice more with the dilute alkaline phosphate buffer. After this it is suspended with 200 ml. of water and kept in the ice chest.

Fructose-1,6-diphosphate solution is prepared by dissolving 325 mg. of potassium sulfate in a few ml. of water and adding this to 800 mg. of fructose-1,6-diphosphate (barium salt) in about 35 ml. of water. After stirring well one adds a few drops of phenol red and enough 1 *N* sodium hydroxide to bring to neutrality. The preparation is filtered, diluted to 50 ml., preserved with toluene, and kept in the ice chest.

The apozymase suspension containing triosephosphates is prepared by digesting 5 ml. of the fructose-1,6-diphosphate solution with 10 ml. of the apozymase suspension at 25° C. for 25 minutes. The preparation is then kept in an ice bath. It will remain serviceable for 2 or 3 hours.

Phosphate buffer of pH 8.0 is prepared by mixing 5 ml. of 0.5 *M* KH_2PO_4 to 250 ml. of 0.5 *M* Na_2HPO_4.

In this experiment the aldolase of the washed yeast splits the fructose-1,6-diphosphate to dihydroxyacetone phosphate and (*d*)-3-phosphoglyceric aldehyde. This latter is phosphorylated to form (*d*)-1,3-diphosphoglyceric aldehyde. This compound is oxidized to form (*d*)-1,3-diphosphoglyceric acid if diphosphoglyceric aldehyde dehydrogenase is present. Since the apodehydrogenase is present in the yeast preparation, only coenzyme 1 need be added. The hydrogen removed from the aldehyde serves to reduce the methylene blue to leuco methylene blue.

Diphosphoglyceric aldehyde dehydrogenase is inactivated by 0.001 *M* sodium iodacetate. Try this.

247. The Estimation of Coenzyme 1 from the Volume of Oxygen Used (Krishnan, P. S., *Science* **105**, 295 [1947]). Prepare washed yeast by stirring 10 g. of quickly dried brewer's bottom dried yeast with 800 ml. of distilled water at the ordinary temperature and then centrifuging in the cold room for 30 minutes. The residue is suspended in 400 ml. of 0.2 *M* phosphate buffer, pH 8.0 and stirred mechanically at room temperature for 30 minutes. The mixture is centrifuged in the cold room for 30 minutes and the washing with the buffer is repeated once more. The residue is then washed twice with distilled water. At this stage the centrifuging has to be carried out for at least 45 minutes. The final residue is stirred with a small quantity of water and transferred to a measuring cylinder. The volume of the suspension is made up to 50 ml., so that each ml. of the suspension would correspond to 200 mg. of the dried yeast. The pH is about 6.4 Store frozen.

This washed yeast suspension is used with the following test system, in the Warburg apparatus, to measure oxygen uptake.

Side arm: 0.1 ml. of methylene blue, 1 in 100.
0.1 to 0.4 ml. of coenzyme, containing 4 to 16 γ.
Water added to make up to 0.5 ml.

Main flask: 1 ml. of the yeast suspension.
0.5 ml. of Harden and Young ester.
0.3 ml. of 0.5 *M* phosphate buffer, pH 8.0.
0.1 ml. of Mg-Mn mixture, containing 0.5 mg. of each.
(The addition of this solution is optional.)
Water added to make up to 2.3 ml.

Central cup: 0.2 ml. of 20 per cent KOH.
Total volume 3.0 ml.

The bath temperature should be 38° C. The flasks should be oscillated at the rate of 100 to 125 strokes per minute.

The flasks are allowed to equilibrate with shaking for 15 minutes in the bath, during which time the aldolase splits hexosediphosphate into triosephosphates. The coenzyme solution and methylene blue are now tipped from the side arm and the flasks returned to the bath and the shaking continued. After 3 more minutes, when the flasks have regained equilibrium, the shaking is stopped and the zero readings noted. The flasks are then shaken for a period of exactly 15 minutes and the readings are observed again. The oxygen absorption in micro-liters during the first 15 minutes, after correcting for blank absorption, is plotted against the coenzyme concentration.

Harden and Young Ester. 1.6 g. of the barium salt of fructose-

diphosphate and 1.0 g. powdered potassium sulfate are ground together in mortar with about 75 ml. of water. A few drops of phenol red indicator are added and the mixture is neutralized by the addition of about 3 ml. of 5 per cent sodium hydroxide. The mixture is centrifuged and the supernatant is made up to a volume of 100 ml. and stored in the cold room after adding a few drops of toluene. The solution is stable for a period of several weeks.

0.5 M Phosphate buffer, pH 8.0. 250 ml. of 0.5 *M* Na_2HPO_4 is mixed with about 12 ml. of 0.5 *M* KH_2PO_4 and the pH is checked with the glass electrode.

USEFUL FIGURES

If 75.4 g. of solid ammonium sulfate are dissolved in 100 ml. of water the solution will be saturated at 20° C. The volume will be 140 ml. and the percentage of ammonium sulfate will be 53.8.

The pH of 0.05 *N* acid potassium phthalate at 20° C. is 4.00.

DESK EQUIPMENT—BIOCHEMISTRY LABORATORY

Important: This is a complete list of the apparatus in every desk. Check your desk and report any broken or missing pieces. When these are supplied, any further requests for equipment will mean charging the material against your breakage fee.

TOP DRAWERS

20 test tubes, pyrex
4 stirring rods
1 thermometer
4 watch glasses: 1– 50 mm.
2– 75 mm.
1–125 mm.
1 test tube holder
1 test tube brush
1 china marking pencil
1 box matches
1 file
1 evaporating dish 18 cm.
1 evaporating dish 8 cm.
1 porcelain test plate
100 sheets 18 cm. filter paper
100 sheets 11 cm. filter paper
1 sq. yard cheese cloth
1 graduated cylinder 50 ml.
1 Mohr pipette, graduated
6 transfer pipettes: 1 ml.
2 ml.
5 ml.
10 ml.
25 ml.
50 ml.
2 1 ml. Ostwald pipettes
1 2 ml. Ostwald pipette
3 Folin-Wu sugar tubes
2 digestion tubes, graduated at 35 and 50 ml.
2 digestion tubes, not graduated
2 200 ml. volumetric flasks
2 100 ml. volumetric flasks
1 Kjeldahl distilling tube
1 burette
1 micro Bunsen burner
1 rubber policeman
1 crucible tongs

CABINET

1 iron ring stand
2 clamps and holders
2 rings
1 filtering arm
1 tripod
1 wire gauze
1 test tube rack
1 sponge
2 Bunsen burners
Rubber tubing for burners
1 wash bottle (1 liter)
1 500 ml. Florence flask
2 Kjeldahl flasks
2 bottles
8 Erlenmeyer flasks: 2 125 ml.
4 250 ml.
2 500 ml.
8 beakers: 2 100 ml.
2 250 ml.
2 400 ml.
2 600 ml.
3 funnels: 2 65 mm.
1 100 mm.
2 pyrex centrifuge bottles, 250 ml.
2 pyrex lipless centrifuge tubes, 50 ml.

This apparatus cost about $27.00 in the U. S. A. in 1944.

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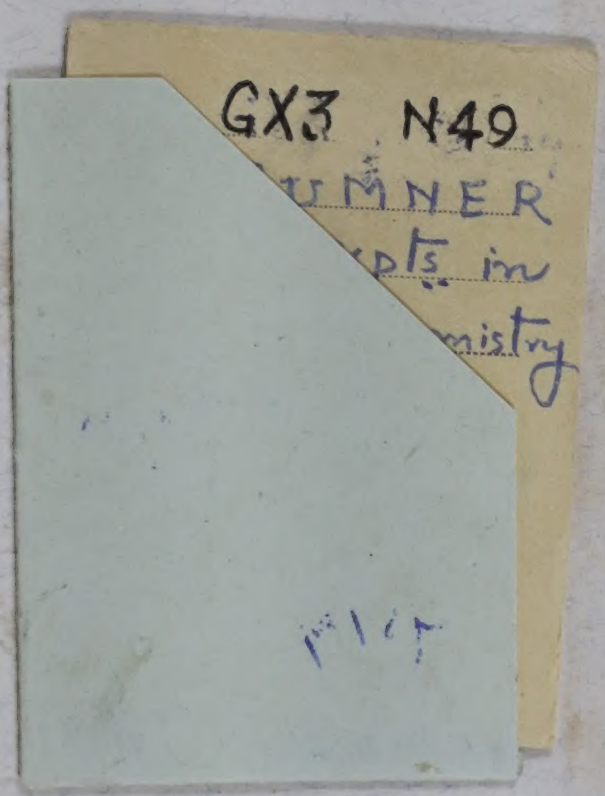
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